ARBUSCULAR MYCORRHIZAL FUNGI AS A BIO-INDICATOR OF SOIL HEALTH UNDER AGRICULTURAL MANAGEMENT PRACTICES IN SOUTH AFRICA

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ABSTRACT

This study investigated the activity of arbuscular mycorrhizal (AM) fungi as a potential biological indicator of soil health under conventional and conservation agricultural management in South Africa. An experimental trial consisting of three replicates plots under conventional and reduced tillage subdivided into twelve treatments of six crops and two fertilizer inputs was assessed over four growing seasons for various AM fungal parameters such as spore density, most probable number (MPN) of propagules percentage root colonisation and easily extractable glomalin (EEG). Cropping combinations were maize monoculture; maize soybean rotation; maize cowpea rotation; maize cowpea intercropping; maize oats intercropping and maize vetch intercropping.

Resident AM fungal spore numbers and EEG protein levels were very low and no root colonization was recorded in the first two growing seasons. These findings prompted the need for the inoculation of the study site in the third growing season with a commercial AM fungal product (Mycoroot^{TM}). Spore numbers, EEG concentrations and percentage root colonisation increased 8 weeks after inoculation but were significantly reduced in the fourth growing season that was not inoculated. MPN infectivity increased with inoculation particularly under conventional tillage and maize monoculture. Resident spore taxa were morphologically identified into three genera *Gigaspora*, *Scutellospora,* and *Glomus*. For the first two growing seasons, the maize roots were heavily colonized by a pathogenic fungus after mycorrhizal inoculation no evidence of pathogenic fungi was observed. In the fourth growing season which did not receive inoculation, root colonization started to decline.

Reduced tillage, high fertilizer input combined with maize cowpea rotation (MC) and maize hairy vetch intercropping (Mv) had a significant effect (P = 0.01) on AM fungal spore numbers. Cropping systems and high fertilizer input had a significant effect on EEG concentrations in the second growing season. Overall, fertilizer application and crop type had implications for mycorrhizal activity. The soil health status in this study site was deemed low as measured by the impaired mycorrhizal activity due to agricultural management practices. Field inoculation combined with classical and molecular tools could provide a more realistic assessment of the effect of agricultural management practices on AM fungi as potential bioindicators of soil health. Therefore, AM fungi could be used as bioindicators of soil health under agricultural management practices in South African soil conditions.

DECLARATION

I the undersigned hereby declare that I am the sole author of the thesis titled arbuscular mycorrhizal fungi as a bio-indicator of soil health under agricultural management practices in South Africa. This is my original work and all source of information quoted and used are acknowledged incomplete references. This thesis has not been previously entirely, or in-part submitted to any other university for a degree.

Wendy Maphefo Sekgota

Signature_________________ Date 29 August 2018

DEDICATIONS

To the Lord and my entire family for your endless love, support and guidance through thick and thin, ups and downs of my life.

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LIST OF ABREVIATIONS

<	less than
>	greater than
µ	microns (10^{-6} of what)
AM	arbuscular mycorrhizal
ANOVA	analysis of variance
BSA	bovine serum albumin
MPN	most propable number
DGGE	denaturing gel gradient electrophoresis
DNA	deoxyribose nucleic acid
e.g.	for example
et al	and others
ELISA	enzyme-linked immunosorbent assay
INVAM	international collection of vesicular-arbuscular mycorrhizal fungi
Mg/ml	milligram/millilitre
MHB	mycorrhiza helper bacteria
P	phosphorus
PCR	polymerase chain reaction
PGPR	plant growth promoting rhizobacteria
PGPF	plant growth promoting fungi
PLFA	phospholipid fatty acid
PVLG	polyvinyl lactophenol glycerol
RNA	ribose nucleic acid
rDNA	ribosomal deoxynucleic acid
rpm	rotation per minute
rRNA	ribosomal ribonucleic acid
SD	Standard Deviation
SOM	soil organic matter
sp.	species (singular)
spp.	species (plural)
SSU	small subunit
SIR	substrate induced respiration
T-RFLP	terminal restricted fragment length polymorphism
w/v	weight/ volume
v/v	volume/volume

1. LITERATURE REVIEW

1.1 MYCORRHIZAL FUNGI

Mycorrhiza is a term that refers to a mutualistic symbiotic association between beneficial soil-borne fungi and plant roots (Quilambo, 2003, Smith and Read, 1997). The term mycorrhiza is a combination of two Greek words, *mycos* meaning "fungus" and *rhiza* meaning "root". This term was established over 100 years ago by a German scientist A.B. Frank in 1885 (Hawksworth *et al.*, 1995). Fossil evidence shows that mycorrhizal fungi were instrumental in the colonization of land by plants. This evidence, collected from molecular sequence data of the divergent 18S rDNA gene, showed that mycorrhizal fungi appeared about 400 to 460 million years ago between the Ordovician-Devonian periods (Pivato *et al.*, 2008, Remy *et al.*, 1994). Mycorrhizal fungi colonise roots of 90% of all plant species (Strack *et al.*, 2003). These fungi are important because they play key roles in the ecology of natural and agricultural systems (Habte, 2006). The association of mycorrhizal fungi with plants is broadly divided into two groups namely ecto- and endomycorrhiza. These are further divided into seven main types based on the morphological structure that the fungi form within and around the host roots. Some of these mycorrhizal fungi are host specific and some generalists. The seven mycorrhizal types are namely orchid, ericoid, arbutoid and monotropoid mycorrhizas, ectendomycorrhiza, ectomycorrhiza, and arbuscular mycorrhiza (Brundrett, 2004) (Fig 1.1).

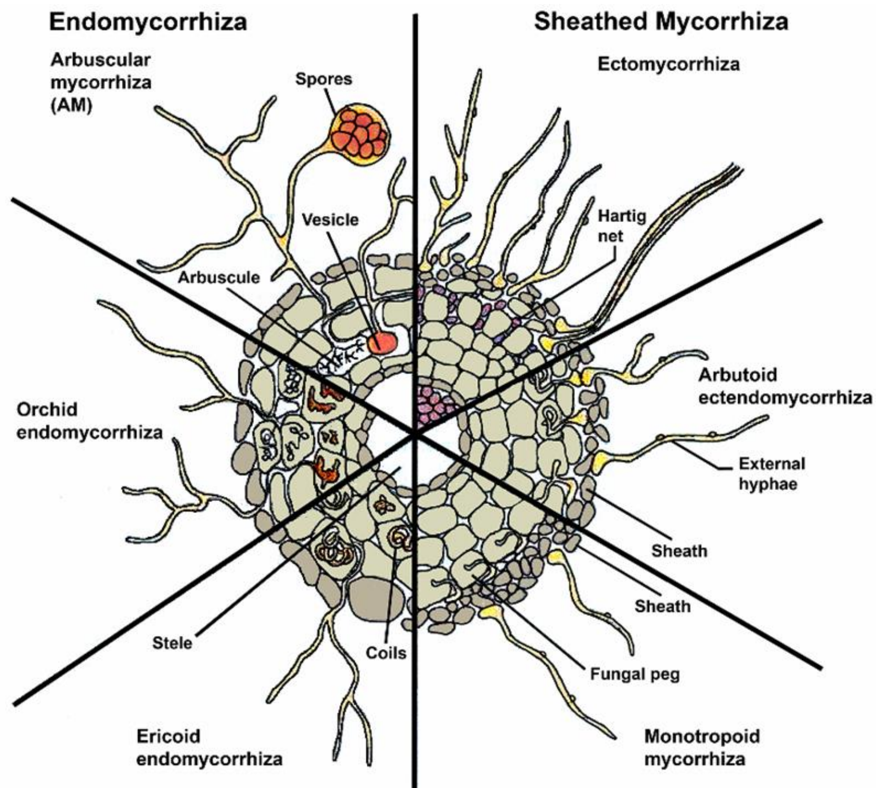

Figure1.1 Cross section of a plant root showing different types of mycorrhiza and the structures they use to form association with their host (Brundrett, 2004; Prescott *et al*., 2005)

1.2 TYPES OF MYCORRHIZAL ASSOCIATIONS

Ericoid mycorrhizal fungi form endomycorrhizal associations (Read, 2002) with approximately 3400 plants species belonging to the plant families Ericaceae, Empetraceae, and Epacridaceae of the order Ericales and species of Pyrolaceae family (Peterson and Massicotte, 2004, Tsuyuzaki *et al*., 2005). The fungi belong to the phylum Ascomycota, with species such as *Hymenoscyphus ericae* being the most extensively studied. Ericoid fungi are host specific and favour plants that grow in poor nutrient soils, with pure stands such as dwarf heath shrubs. The association is characterised by coiled structures that colonize the cell wall of fine roots (Fig 1.1) (Finlay, 2007, Peterson and Massicotte, 2004). In South Africa, these mycorrhizas form an association with the fynbos plants of the acidic sandy soils of Southern and Western Cape (Mitchell and Gibson, 2006, Stracker and Mitchell, 1987, Stracker, 1996).

Orchid mycorrhizal fungi form endomycorrhizal associations with plant species belonging to the Orchidaceae family. These fungi belong to Ascomycota and Basidiomycota phyla and are host specific they are recognised by the formation of coils or pletons inside cortical cells (Fig 1.1) (Finlay, 2007, Harley, 1969, Peterson and Massicotte, 2004). This mycorrhizal type is not well researched in South Africa.

Monotropoid mycorrhizal fungi form ectomycorrhizal associations with plants species belonging to the Monotropaceae family. These fungi belong to the Basidiomycota phylum and are host specific (Read, 2002). They develop a mantle and superficial 'Hartig' net, with single hyphae growing into the epidermal cells forming peg-like structures (Fig 1.1) (Finlay, 2007, Peterson and Massicotte, 2004).

Arbutoid mycorrhizal fungi form ectomycorrhiza associations with plants species in the Pyrolaceae family, mostly with *Arctous*, *Arbutus* and *Arctostaphyl* (Finlay, 2007). These mycorrhizal fungi are host specific and they establish their association with their host by forming an extensive intracellular fungal proliferation (Fig 1.1) (Finlay, 2007, Peterson and Massicotte, 2004, Read and Stribley, 1975).

Ectendomycorrhizas have characteristics that are similar to endo and ectomycorrhizas and are often called E-type mycorrhizas. The fungi forming these associations are mainly from the Ascomycota and their hosts mostly include *Pinus* and *Larix* species. These fungi start their association as ectomycorrhizas and as they mature and change to form endomycorrhizas (Fig 1.1) (Finlay, 2007).

Ectomycorrhizal fungal associations are formed with terminal feeder roots of woody perennial plants (Taylor and Alexander, 2005). The fungal species are mostly from the phylum Basidiomycota, few species of the phylum Ascomycota (Finlay, 2007) and rarely from Zygomycota (Hawley and Dames, 2004). This is the second most widely spread mycorrhizal type in the world, forming associations with approximately 8 000 plant species (Fischer, 2007). They are dominant in tropical forests and temperate regions (Hawley and Dames, 2004). These fungi may reproduce by forming sporocarps containing a high concentration of nitrogen (N), phosphorus (P) and micro-nutrients (Taylor and Alexander, 2005) because they are good transporters of N and P in nutrient-poor soil environment (Tsuyuzaki *et al.*, 2005). The ectomycorrhizal association is characterized by the formation of a mantle or outer sheath and a 'Hartig' net which is an intracellular network of hyphae within extra-radial hyphal networks and rhizomorphs (Fig 1.1) (Hawley and Dames, 2004).

Arbuscular mycorrhizal (AM) fungi are symbiotic endomycorrhizal fungi forming an association with approximately 250,000 host plants (Franken and George, 2007). Their hosts plants range from hosts such as bryophytes, gymnosperms, gametophytes, halophytes, hydrophytes, xerophytes, lycopods, and angiosperms as well as non-root tissue plants like pteridophytes and Psilotales (Quilambo, 2003). AM fungi are the focus of this research, and the association is described further in the following sections.

Plant species of the families like Chenopodiaceae (Gosling *et al.*, 2006), Proteaceae, Cruciferae, Zygophyllaceae, Amaranthaceae, and Cryperaceae generally are non-mycorrhizal but rare associations can occur under stressful growth conditions (Quilambo, 2003).

1.3 ARBUSCULAR MYCORRHIZAL FUNGI

Arbuscular mycorrhizal (AM) fungi are common in most environments (Johansson *et al.*, 2004). They occupy a wide variety of terrestrial, aquatic and even extreme environments (Danesh *et al.*, 2006). They are the only mycorrhizal type that forms symbiotic associations with most agriculture and horticultural crops (Franken and George, 2007). Their distribution and occurrence is influenced by natural and land use factors such as soil moisture, pH, and temperature, mining and agricultural practices (Jansa *et al.*, 2008). AM fungi are very sensitive to soil disturbance, hence any changes to the soil will influence the life cycle. They are recognised by the formation of finely branched arbuscules within cortical root cells and intercellular hyphal growth which may produce lipid-filled vesicles (Fig 1.1; Fig 1.2) (Hawley and Dames, 2004).

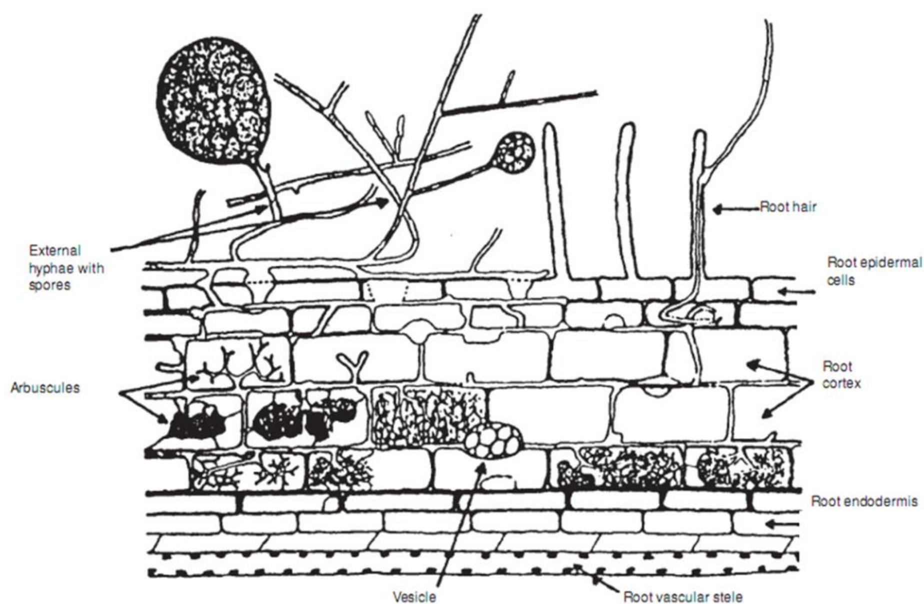

Figure 1.2 Arbuscular mycorrhizal (AM) fungal structures formed inside and outside when in associated with host plant roots (Habte, 2000).

Arbuscular mycorrhizal fungi are obligate symbionts that depend on colonisation of their host for completion of their life cycle. These fungi reproduce asexually by producing propagules such as spores (40 - 800 µm) that are bigger than other fungal species. Colonised root

fragments, soilborne auxillary vesicles (some genera) and extra- as well as intra-radical hyphae serve as a source of inoculum for the completion of their life cycle (Jasper *et al.*, 1989). The presence of these propagules can be used as a measure of the activity and infectivity of these fungi in the soil (Bedini *et al.*, 2007, Castillo *et al.*, 2006). Any changes in the propagule numbers can be a useful marker to indicate changes in the soil biological, chemical and physical properties (Aggarwal *et al.*, 2010, Castillo *et al.*, 2006, Mathimaran *et al.*, 2007). However, hyphal fragments and colonized root fragments survive for a short period without a suitable host. Some members of AM fungi depend solely on intact hyphal networks and root fragments to establish colonization, especially members of the family Glomeraceae (Schalamuk and Cabello, 2010). In disturbed soil, spores can be the main source of inoculum because they survive longer in the soil even under unfavorable environmental and human-induced conditions (Castillo *et al.*, 2006, Kabir, 2005). The start of the life cycle of AM fungi with spores as a source of inoculum is known as the asymbiotic phase.

1.3.1 Asymbiotic and pre-symbiotic phase

AM fungal spores germinate in response to environmental conditions such as pH, moisture, CO_2, and root exudates released in the presence of a suitable host plant. This phase is host independent. However, spores germinate in different forms that can be used in their morphological identification and taxonomy. Spores can germinate by forming a germ tube through the papillate layer, or by penetrating directly through the spore wall or through newly formed spore walls by forming flexible inner germination walls, shields or orbs. Others germinate by forming membranous structures in the outer spore wall such as a terminal swollen sac (mother spore, vesicle, hyphal terminus or sporoferous saccule) from single hyphae either as a single spore or in a sporocarps (spore-bearing spore sac). These 'sporiferous saccules' can collapse and disappear during spore maturation (Gerdemann and Trappe, 1974, Morton and Benny, 1990). Some genera germinate laterally on or in subtending hyphae of the sporogenous vesicle by hyphal swelling (Trappe and Schenck, 1982, Walker, 1992). In the absence of a suitable host and the recognised plant-derived signals, germination will cease after 25-30 days, depending on the species or genera of AM fungi several unsuccessful germination attempts can be made (Giavannetti and Sbrana, 1998, Reguena *et al.*, 2007). In the presence of a suitable host, volatile, hydrophobic metabolites and constitutes of root exudates such as flavonoids are released to signal and trigger hyphal growth from germinated spores and also serve as a source of food, this initiates the pre-symbiotic phase. When the hyphae are triggered it will grow toward a host plant to initiate adhesion (Gianinazzi-Pearson, 1996, Pinior *et al.*, 1999, Raaijmaker *et al.*, 2009). On contact with their host, these fungi may form appressoria. The penetration of the root cell wall can occur either directly or from a penetration peg (Fig 1.2). After penetration the hyphae colonize the root cortical region

of host by intercellular and intracellular hyphae in the symbiotic phase. But, the rate of colonization is dependent on a number of factors such as host species, AM fungal species, soil and environmental conditions such as pH, moisture, CO_2, and management practices. As a result, these factors can delay the start of the life cycle of these fungi by days, weeks and even months (Garcia-Garrido *et al.*, 2000, Reinhardt, 2007). Successful colonization of the host plant is known as symbiotic phase.

1.3.2 Symbiotic phase

During the symbiotic phase AM fungi use two types of colonization strategies namely 'Paris' type characterized by forming hyphal coils inside cortical cells and 'Arum' type characterized by forming fine branched hyphal structures called arbuscules inside the cortical cells. The formation of these structures can aid in the identification and taxonomy of these fungi to species level. These structures also serve as a site for nutrient exchange between host and fungus (Finlay, 2007, Muthukumar and Prakash, 2009). Some genera form vesicles which are globose, lipid-rich structures inside the cortical cells of host plants (Fig 1.2) and others form soil-borne auxillary cells instead. During this association, extra-radical hyphae will form a network that connects roots to the rhizosphere. This network exploits, absorbs and transfers nutrients and water from the soil for their host and in exchange for carbohydrates to produce new reproductive structures (Finlay, 2007, Rillig, 2004).

During host death (usually at the end of the plant growth cycle) AM fungi produce extra-radical hyphae, spores, auxillary cells and colonized roots fragments that serve as propagules to ensure the continuation of the AM life cycle and this is sometimes referred to as the exploratory or dying phase (Finlay, 2007). Spores can vary in shape from globose to subglobose, ovoid, obovoid, and even irregular. Different spore forms of AM fungi such as glomoid, gigasporoid, scutellosporoid, entrosporoid, acaulosporoid, entrophosporoid and pacisporoid can be used to distinqued one genus from another providing a means of morphological identification of these fungi (Morton, 1988, Morton and Benny, 1990, Oehl *et al.*, 2011 a, b & c). These spores can have two and even more layers that differ in size, color, refractivity, flexibility, histological reactivity, and ornamentation. Spores can have a thin outer, flexible inner, hyaline layer that sloughs, or variable number of laminae layers which form as the spore matures and a papillate layer formed prior to germination, extending into the lumen of the spore. Auxillary cells are also used to distinguish one genera from another, this soil borne structures can be lumpy, knobby, and spiny or papillate in shape (Douds and Millner, 1999). Spores survive longer in soil than other propagules and can serve as identification tools that aid in the taxonomy of AM fungi, distinguishing genera and species one from another (Jansa *et al.*, 2003).

1.3.2 Classification of AM fungal taxa

The classification of AM fungi has evolved since the taxonomy started by the description of the genus *Endogone* Link: Fries with one species *Endogone pisiformis* that had minute globose sporidia, with small and uniform size zygospores. Tulasne and Tulasne, (1845) described two species *Glomus microcarpus* and *G. macrocarpus*, Tul. & Tul 1845 that had terminal spores, borne on vegetative hyphae that resembled chlamydosporic species. Tulasne and Tulasne (1851) placed *G. microcarpus* and *G. macrocarpus* into the genus *Endogone* with the addition of two species *Endogone pisiformis* and *E. lactiflua*. Re-evaluation of taxonomy of these fungi by Bresadola in 1896 revealed that there were three types of spores namely zygospores, sporangiospores, and chlamydospores which were used as keys in taxonomy. These keys aided Bucholtz (1912), in moving the family Endogonaceae into the order Endogonales (Morton, 1988). Thaxter (1922) used these taxonomic keys to describe the genus *Sphaerocreas* with one species *Sphaerocreas pubescens* Sacc. & Ellis that was later transferred into the genus *Endogone*. Peyronel (1924) was the first person to properly identify these fungi as vesicular-arbuscular mycorrhizal fungi based on sporocarps containing several chlamydospores (Gerdemann and Trappe, 1975, Morton, 1988).

Taxonomic studies of AM fungi by Gerdemann and Trappe (1975), Morton (1988), Trappe and Schenck (1982) further separated the Endoganaceae family into seven genera: *Acaulospora* Gerd. & Trappe, *Endogone*, *Gigaspora* Gerd. & Trappe, *Glaziella* Berk., *Modicella* Kanouse, *Sclerocystis* Berk. & Broome and *Glomus* (Tulasne & Tulasne, 1845). These studies provided enough evidence for these fungi to be moved into the phylum Zygomycota, with class Zygomycetes (Table 1.1) (Morton, 1988, Warcup 1990, Walker, 1992). These fungi were also called vesicular-arbuscular mycorrhizas (VAM) based on the formation of vesicles by four out of six genera *Sclerocystis, Glomus, Acaulospora,* and *Entrophospora*. But this name was later changed to arbuscular mycorrhizal (AM) fungi because *Gigaspora* and *Scutellospora* do not form vesicles, but form soil borne auxillary cells (Gerdemann and Trappe, 1974, Morton, 1988, Morton and Benny, 1990).

In South Africa AM fungi were first reported by Hattingh in 1972 after he described a honey coloured sessile spore that he assigned to the genus *Endogone sensu lato* of the Endogonaceaous. Coetzee (1982) found spores of AM fungi in the rhizosphere soil of maize (*Zea mays* L.) from Fouriesburg district of the Orange Free State. Hoffman and Mitchell (1986) found chlamydospores of the genus *Glomus* (Tulasne & Tulasne) and three types of *Gigaspora* (Gerd. & Trappe) with auxillary cells from root systems of both alien and indigenous legume species in the South Western Cape Province.

Taxonomy of AM fungi based on molecular techniques subsequently classified these fungi into a monophyletic phylum called Glomeromycota C. Walker & Schuessler that contained 3 families and 6 genera (Table 1.1; Schüßler *et al.*, 2001a). Schüßler *et al.*, (2001a & b) also proposed new families formerly included in Glomeraceae, *Glomus*-Group A (GlGr) or *Glomus*-Group B (GlGr) and the *Diversisporaceae* fam. ined. *Glomus* was divided into three clades A, B, and C (Redecker and Raad, 2006, Schüßler *et al.*, 2001b). The *Glomus* groups Aa with *G. mosseae* and *G. geosporum* was placed in the genus *Funnelisformis* with *F. mosseae* (Nicolson and Gerdemann) and C Walker*, F. coronatus* (Giovann.) C Walker & A Schuessler*, F. caledonis* and *Funnelisformis* sp. WUM3. *Glomus* group Ab along with *G. intraradices* and *G. irregular* were placed in the genus *Rhizophagus* Pirozynsky & Dalpé and *Sclerostis* (*R. irregularis, R. intraradices* and *R. proliferus*). *Glomus* sp. W3347 now known as *G. hoi* was placed in *Glomus* group Ac (S.M. Berch & Trappe). *G. etunicatum* now known as *Claroideoglomus* (C. Walker & Schuessler, 2010) with *C. lamellosum* basionym *G. lamellosum* were placed into *Glomus* group B (Dalpe, Koske & Tews) C. Walker & Schuessler (Krüger *et al.*, 2012, Oehl *et al.*, 2011 a, b, & c, Schüßler and Walker, 2010).

Currently, group Aa1 is genus *Funneliformis*, Aa2 is genus *Simiglomus*, Aa3 is genus *Septoglomus* Sieverd., GA. Silva & Oehl, gen, nov. Group Ab1 is now genus *Glomus*. Group B1 is genus *Claroideoglomus* and B2 is genus *Viscospora.* Group Ab2 is genus *Rhizophagus*, and Ab3 is genus *Sclerocystis*. Group Ca is genus *Diversispora* and Cb genus *Redecker* C. Walker & Schuessler (2010) (Krüger *et al.*, 2012, Oehl *et al.*, 2011 a, b, & c). Oehl *et al.*, (2011 a & b) moved genus *Paraglomus* J.B. Morton & D. Redecker into a new order *Paraglomerales* with one family *Paraglomeraceae* (Table 1.1). Genus *Diversispora* (C. Walker & A. Schuessler) was moved into a new order *Diversisporales* C. Walker & Schuessler, with one family *Diversisporaceae* C. Walker & Schuessler, fam. nov. *Glomus occultum* and *G. brasilianum* are now grouped into a new genus *Paraglomus,* together with several other species of *Glomus* (*G. callosum*, *G. fecundisporum, G. leptotichum*) have been transferred into the order *Archaeosporales* that has one family Ambisporaceae (Oehl *et al.*, 2011 a, b, & c, Stürmer, 2012) (Table 1.1). The genus *Diversispora* were moved to the new order Diversisporales with three families Acaulosporaceae, Diversisporaceae, and Entrophosporaceae (Table 1.1). *Paraglomus* is classified under the new order Paraglomerales with one family Paraglomeraceae. *A. colombiana* and *A. kentinensis* formerly known as *Kuklospora colombiana* and *K. kentinensis* are accepted as new combinations (Oehl *et al.*, 2011 a & b, Krüger *et al.*, 2012, Stürmer, 2012). These systematic changes can be confusing and in 2011 at the International Conference of Mycorrhizas (ICOM 7) in India, Krüger *et al.*, (2012) presented the now recognised, rationalised taxonomy which included the Glomerales

order consisting of two families Glomeraceae and Claroideoglomeraceae (Table 1.1) (Oehl *et al*., 2011 a & b). However, taxonomic revisions are likely to continue as both morphological and molecular information increases, the use of Operation Taxonomic Units as used in the Maarjam sequence database has been suggested (Krüger *et al*., 2012).

Table 1. Classifications and revision of Glomeromycotan fungi since 1974 to present (Stürmer, 2012).

Phylum	**Class**	**Order**	**Family**	**Genera**	**Authorities**
Zygomycota	Zygomycetes	Endogonale	Endogonaceae	*Glomus*	Gerdemann and Trappe, 1974
				Sclerosystis	
				Acaulospora	
				Gigaspora	
Zygomycota	Zygomycetes	Glomerales	Glomeroceae	*Glomus*	Morton and Benny, 1990
				Sclerocystis	
			Acaulosporaceae	*Acaulospora*	
				Entrophospora	
			Gigasporaceae	*Gigaspora*	
				Scutellospora	
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	*Glomus*	SchÜßler *et al* ., 2001
		Diversisporales	Gigasporaceae	*Gigaspora*	
				Scutellospora	
			Acualosporaceae	*Acaulospra*	
				Entrophospora	
			Diversisporaceae	*Diversispora*	
		Paraglomerales	Paraglomaceae	*Paraglomus*	
		Archaeosporales	Archaeosporaceae	*Archaeospora*	
			Geosiphonaceae	*Geosiphon*	
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	*Glomus*	Walker and SchÜßler, 2010
				Funnelisformis	
				Sclerocystis	
				Rhizophagus	
			Chlaroideoglomeraceae	*Chlaroideoglomus*	
			Gigasporaceae	*Gigaspora*	
				Racocetra	
				Scutellospora	
			Acaulosporaceae	*Acaulospora*	
			Entrophosporaceae	*Entrophospora*	
			Pacisporaceae	*Pacispora*	
			Diversisporaceae	*Diversispora*	
				Otospora	
				Redeckera	
		Paraglomerales	Paraglomeraceae	*Paraglomus*	
		Archeaosporales	Archaeosporaceae	*Archaeospora*	
			Ambisporaceae	*Ambispora*	
			Geosiphonaceae	*Geosiphon*	
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	*Glomus*	Oehl *et al* ., 2011 a & b
				Funneliformis	
				Simiglomus	
				Septoglomus	
			Clariodeoglomeraceae	*Clariodeoglomus*	
				Viscospora	
		Diversisporales	Diversissporaceae	*Diversispora*	
				Redeckera	
				Otospora	
			Entrophosporaceae	*Entrophospora*	
			Acaulosporaceae	*Acaulospora*	
				Kuklospora	
				Pacispora	
		Gigasporales	Gigasporaceae	*Gigaspora*	
			Scutellosporaceae	*Scutellospora*	
				Orbispora	
			Racecentraceae	*Racocetra*	
				Cetraspora	
			Denstiscutataceae	*Dentisscutata*	
				Fuscutata	
				Quatunica	
	Archaeosporomycetes	Archaeosporales,	Archaeosporaceae	*Archaeospora*	
				Intraspora	
			Ambisporaceae	*Ambispora*	
			Geophonaceae	*Geosiphon*	
	Paraglomeromycetes	Paraglomerales	Paraglomeraceae	*Paraglomus*	

1.3.3 Identification of AM fungi

Estimation of AM fungal activity and population can be determined by extraction of propagules such as spore and hyphae from soil samples. They can be estimated by wet sieving and decanting, in combination with plate methods, adhesion-flotation, sucrose centrifugation, gelatine column, airstream fractionation, and fixing soil slurries to filter paper. After extracting propagules from the soil, they are further identified by microscopy to distinguish one genera from another by using morphological characteristics such as color, shape, germination type and even hyphal attachments. However, the identification of field collected spores that are parasitized or damaged is difficult (Bago *et al.*, 2004, Guadarrama *et al.*, 2008). Jansa *et al* (2002), argued that the assessment of AM fungal spores directly from field samples is the most efficient approach to studying their community. However, morphological keys used to identify AM fungal spores have constraints. Few genera can be distinguished using these keys, because of the limited characteristics. Morphological keys require time because they are laborious, and, in some cases, identification can only be made to genus level. According to Mathimaran *et al* (2007), the assessment of spores in the soil alone does not give an indication of the activity of these fungi. Proper identification of these fungi requires pure cultures that are produced by trap culture with a highly mycorrhizal host in complicated and complex substrates because they are not culturable on artificial medium. However, *Jansa et al* (2002) suggest that trap cultures should be established with several host plant species to avoid the selective effect of host plants. Growth of AM fungi in aeroponics or hydroponic approaches and monosporic cultures with transformed carrot roots have also been used with limited success to produce spores that are more easily identified (Bi *et al.*, 2004, Fortin *et al.*, 2002, Francchia *et al.*, 2001).

AM fungi inside host root cells can be determined through staining and observation of roots by light microscopy which is a standard method that is widely used to study the activity of these fungi (Veirheilig *et al*, 2005, McGonigle *et al.*, 1990). The measurement of activity and degree of root colonization through the visual observation of typical AM fungal structures such as arbuscules, hyphal coils, vesicles and auxiliary cells is required (Redecker and Raad, 2006). The structures are observable by staining roots with either non-vital stains (i.e. trypan blue, cotton blue, acid fuchsin or ink-vinegar and chlorazol black E) or vital stains (neutral red dye) (Fyson and Oaks, 1992, Veirheilig *et al*, 2005). These stains are used in combination with chemicals like potassium hydroxide (KOH), chloral hydrate and hydrogen peroxide (H_2O_2) that remove cell contents to make viewing and visualization easy and clear (Fyson and Oaks, 1992, Koske and Gemma, 1989, Kosuta *et al.*, 2005, Veirheilig *et al* 1998 a & b, 2005). However, some non-mycorrhizal fungi such as *Olpidium* spp, produce vesicles like structures

and *Polymyxa graminis* produce arbuscule-like structures, as well as microsclerotia that can be mistaken for AM fungi if observed only under the dissecting microscope. Percentage colonization determinations therefore usually are conducted using higher magnification on a compound microscope (McGonigle *et al.*, 1990). In the Stellenbosch region of South Africa, root colonisation levels of between 70 % and 90 % were found in vineyards that were previously inoculated with commercial AM fungal inoculum at two farms (Meyer *et al.*, 2005). Furthermore, it is also important to note that different AM fungal species will exhibit differences in infectivity with some species such as those belonging to the *Glomeraceae* colonizing roots within 4 weeks while members of the *Acaulosporaceae* and *Gigasporaceae* are slow colonizers requiring about 8 weeks (Hart and Reader, 2002 b). Studies show that root colonization can be influenced by AM fungal species type, soil type, agricultural practices, crop types and environmental factors such as pH, temperature and soil moisture (Aggarwal *et al.*, 2010, Oehl *et al*, 2010). As a result, certain species are adapted to performing well under certain soil conditions and if any changes occur to this soil conditions, this fungal species will not survive (Aggarwal *et al.*, 2010). Hence, that is the reason for using these parameters to determine the activity of AM fungi that can be linked to the improvement of soil aggregate stability, tilth and can give a measure of the status of soil health.

Current studies of AM fungal have evolved into using both morphological characteristics of spores and molecular techniques to effectively gather species diversity data. Molecular techniques like DNA extraction with polymerase chain reaction (PCR) methods based on the amplification and sequences analysis of small subunit (SSU) of rRNA of the 18, 25, 48 rDNA regions, including ITS regions are commonly used. Other techniques used are denaturing gradient gel electrophoresis (DGGE), restricted fragment length polymorphism (RFLP), single-stranded conformation polymorphism (SCCP), terminal RFLP (T-RFLP), real-time qRT-PCR and temperature gradient gel electrophoresis (TGGE) (Liang *et al.*, 2008, Ma *et al.*, 2005). However, these methods are costly and time consuming. Therefore, AM fungal investigations are still primarily based on the analysis of classical stained roots and percentage colonization assessment and spore morphological characterization and enumeration to provide information on mycorrhizal activity. These fungi are considered to be the most important members of the rhizosphere due to their contributions to plant fitness and soil health (Barea *et al.*, 2005).

1.3.4 Key roles of AM fungi in the soil.

properties (Sylvia, 1992, Wright *et al.*, 1999). Their external hyphal networks entangle and enmesh micro-aggregates (<0.250 mm) and macro-aggregates (>0.250 mm) in the soil,

releasing a compound into the soil that is hydrophobic and acts as a cementing agent (Habte, 2006). These fungal hyphae have the ability to glue substances such as sand, silt, plant debris and organic matter contributing to a major component of soil organic matter (Miller and Jastrow, 2000, Wright *et al.*, 2006).

AM fungi improve soil structure, texture, tilth, aggregate stability, water relations and C deposition in the soil through the production of a brown coloured glycoprotein called glomalin (Bedini *et al.*, 2007, Schloter *et al.*, 2003, Wu et al., 2016). Glomalin is deposited into the soil through extra-radical hyphae and spore walls of AM fungi and their decomposition (Driver *et al.*, 2005, Janos *et al.*, 2008, Rillig, 2004). Some scientists called glomalin "gobs" because it can form a waxy layer. The compound consists of a mixture of carbohydrates and protein, with 1-9% tightly bound iron (Rillig *et al.*, 2002a, Tresender & Turner, 2007). Glomalin is operationally defined as glomalin-related soil protein (GRSP), the term glomalin being reserved for the purified protein or gene that can be a thioredoxin containing chaperone (Gillespie *et al.*, 2011). It can store up to 15 % of fixed carbon in the soil for a period of up to 100 years (Antibus *et al.*, 2006, Lutgen *et al.*, 2003). Glomalin can fall into two categories based on the amount of glomalin extracted, time and cycles needed for extraction. Total extractable glomalin (TEG) is the maximum amount of glomalin that has accumulated over time. Easily extractable glomalin (EEG) is the fraction of glomalin that is recently deposited (Janos *et al.*, 2008, Rillig *et al.*, 2001a & b). Two methods are commonly used to effectively determine EEG and TEG concentration in the soil. The first one is Bradford protein dye binding assay that measures the binding of Coomassie Brilliant Blue G-250 dye to protein as it shifts absorbance from 465-595 nm. This assay is very rapid and reproducible because binding of the dye can take less than two minutes to complete with good colour stability (Bradford, 1976), but it is not specific for glomalin only. The other method is the use of enzyme-linked immunoassay (ELIZA) using monoclonal antibody MAb32B11 specific for AM fungi (Janos *et al.*, 2008). This method is very sensitive and is specific for glomalin.

In the soil glomalin exists as standing stock that is affected by decomposition and production of AM fungal hyphae and spores because it is released in to the soil when dead hyphae and spores walls of AM fungi are deposited and decomposed, the concentration of this protein in the soil can be directly related to the activity of these fungi (Driver *et al.*, 2005, Wright *et al.*, 2006). In other studies, it has been used as a bio-indicator of the presence and activity of AM fungi under different agricultural management practices (Rillig *et al.*, 2003) and could potentially be an indicator of soil health.

1.3.5 Key roles of AM fungi in host plant fitness.

Previous studies show that AM fungi are vitally important in enhancing the uptake of complex and even immobile compounds essential for plant and root fitness. Chemically, (AM) fungi are key in increasing uptake, absorption, and transportation of nutrients for their host plants. They transport nutrients such as copper (Cu), nitrogen (N), sulphur (S), zinc (Z), magnesium (Mg), potassium (K), iron (Fe), calcium (Ca), manganese (Mn), molybdenum (Mo), cobalt (Co) and especially phosphorus (P) by plants (Akond *et al*., 2008, Muok *et al*., 2009). In P poor soils they can regulate the amount of P that the plants extract from the soil (McGonigle *et al*., 1999). For example, Mathur *et al* (2006) study shows that *G. deserticola* and *S. heterogama* increased the uptake of P and N by two-fold in *Plantago ovate* Nels. & Kennedy, a multipurpose medicinal plant under arid and semi-arid conditions. These fungi promoted plant growth of diverse plant communities in soil systems cultivated with soybeans (*Glycine max* L. Merr.) and sorghum (*Sorghum bicolor* L. Moench) (Sylvia *et al*, 1993). Furthermore, AM fungi are intimately linked to their host plant. Therefore, host crop types play an important role in the enhancement of AM fungal activity. However, the effect of cropping sequences is highly dependent on host plant's mycorrhizal status. Crops can be highly mycorrhizal, moderate to non-mycorrhizal (Troeh and Loynachan, 2003). Cropping systems with high mycorrhizal crops can support and maintain a high AM fungal spore population (Kahiluoto *et al*., 2009). Similarly, Kramadibrata *et al* (1995) in Indonesia found a high number of AM fungi within the ranges of 383 to 12 266 spores/ 100g of soil in soybean rhizosphere. However, the effect of cropping practices on AM fungi is also dependent on several factors such as growth season, soil depth and stage of crop development (Aggarwal *et al*., 2010).

Physically, they protect their host against harsh environmental conditions such as drought and salinity. They actively move water into, through and out of host plants grown under stressful conditions which tend to perform better than non-mycorrhizal plants (Audet and Charest, 2007, Boomsma and Vyn, 2008). These fungi use several mechanisms such as improvement and increase in nutrient acquisition, hydration, altered stomatal conductance, transpiration and water uptake to protect and enhance host tolerance to stressful conditions. Several species of *Glomus* sp. such as *G. fasticulatum, G. desertificola, G. intraradices, G. etunicatum, G, mosseae* have been found to be effective in enhancing growth of crops such as lettuce (*Lactuca sativa* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolor*) and alfalfa (*Medicago sativa* L.) grown in areas with harsh environmental and soil conditions (Augé, 2004, Wu and Xia, 2006). Wu and Xia, (2006), found enhanced host tolerance to drought and host osmotic adjustment that originated from K^+, $Ca_2^{+,}$ and Mg_2^+. Subramanian *et al* (2006) found an

increase in drought tolerance of field grown tomato colonized by *G. intraradices* during growth, flowering, fruit production, and fruit harvesting.

Previously studies showed that these fungi can protect their host from absorbing heavy metals from the contaminated environment (Audet and Charest, 2007, Sharma *et al.*, 2007). They increase crop productivity and host tolerance to transplant shock (Turk *et al.*, 2006). In some plants, they increase tolerance to high levels of contaminants and pollutants in metal polluted soil. *For example, Glomus mosseae* and *G. intraradices* enhanced growth of *Helianthus annuus* and tolerance of plants to heavy metals such as cadmium sulfate ($CdSO_4.8H_2O$) and lead acetate ($(CH_3COO)_2Pb.3H_2O$) from polluted soil (Awotoye *et al.*, 2009). On the contrary in another study *G. mosseae* and a mixture of various *Glomus* sp. strains increased the uptake of cadmium (Cd), lead (Pb), strontium (Sr), mercury (Hg) and nickel (Ni) in shoots of the phytostabilising plants such as perennial rye grass (*Lolium perenne* L.), kentucky bluegrass (*Poa pratensis L.*), furrowed fescue (*Festuca rupicola* L.), leaved fescue (*Festuca heterophylla L.*), red fescue (*Festuca rubra* L.) *and* sheeps fescue *(Festuca ovina* L.) in a metal polluted soil (Takács *et al.*, 2005). Increased activities of phosphatase and urease were found in *Elsholtzia splendens* Nakai ex F. Maek and maize inoculation with AM fungi grown in Cu, Zn, Pb and Cd contaminated soil (Wang *and* Qui, 2006). Therefore, it is evident that AM fungi can improve adaptation and growth of plants grown in heavy metal and chemical polluted soil environments. They also improve microbial activity.

Biologically, AM fungi play important roles in the rhizosphere by enhancing microbial activity. The term rhizosphere refers to the physical, chemical and biological zone around the roots that alters microbial diversity and activities. Organic material that is released in this zone is mainly in the form of root exudates that signal and act as growth substrate for microbes. Microbial communities in this zone play a vital role in plant fitness and soil health as they help plants to adapt to stressful conditions such as water and mineral deficiency and soil-borne pathogens. They also interact with saprophytes, deleterious and symbiotic bacteria, fungal pathogens, non-parasitic, minor parasites, and parasitic nematodes (Godeas *et al.*, 1999). The mycorrhizosphere encompasses the rhizosphere and area influenced by the extradical hyphal network (hyphoshere), which encourages interaction with other beneficial microbes such as plant growth promoting fungi (PGPF) and plant growth promoting rhizobacteria (PGPR) (Turk *et al.*, 2006, Schloter *et al.*, 2003). These fungi and their interacting microbial partners can be used as biofertilizers or bio-control agents of soil pathogens. However, mycorrhizosphere microbes can either promote or interfere with mycorrhizal activities (Barea *et al.*, 2002). Positive interactions can occur with phosphate solubilizing microorganisms (PSM), Mn oxidizers and other microorganisms that promote growth of many different species

grown in the floricultural, horticultural (Sinegani and Sharifi, 2007) and forestry industries (Akond *et al*., 2008). Host colonization by AM fungi increases processes such as nitrogen fixation and nodulation of legumes (Kapoor *et al*., 2008), and facilitates the transfer of N_2 derived compounds from legume to non-legumes crops (Van Kessel *et al*., 1985). In a glasshouse experiment conducted by Carling *et al* (1978), the combination of *G. fasciculatus* strains with *Rhizobium japonicum* inoculum on soybean increased the weight of crop, nodulation, level of nitrate and nitrogenase activities.

These AM fungi increase activities of beneficial microorganisms that can in turn influence plant growth. Nogueira *et al* (2007) conducted a study that revealed that inoculation of *G. etunicatum* or *G. macrocarpum* decreased manganese (Mn) oxidising bacteria homologous with genera *Arthrobacter, Variovorax, and Ralstonia.* They also increased Mn reducing bacteria such as streptomycetes in soybean rhizosphere. Inoculation with *G. versiforme* enhanced soil bacterial and actinomycete populations, and improved soil proteinase, polyphenol oxidase, urease, and saccharide activities (Zhao *and* Liu, 2010). Chandanie *et al* (2005) investigated the effect of the interaction of *G. mosseae* and two PGPF strains of the fungus, *Phoma sp*. (GS8-2 and GS8-3) on cucumbers (*Cucumis sativus* L.). They found that dual inoculation of *Phoma* sp. with *G. mosseae* did not influence cucumber growth, but affected root colonization of *Phomas* sp. strains on the host. Dual inoculation of *G. versiforme* and *G. mosseae* with PGBR (B*acillus polymyxa* and *Bacillus* sp.) increased growth of tomato (*Lycopersicon esculentum* Mill) plants (cultivar Jinguan), and controlled root-knot nematodes (*Meloidogyne incognita*). Plant growth especially with dual inoculation of *G. mosseae* and *Bacillus* sp. was increased (Liu *et al*., 2011). Dual inoculation of PGPR *Pseudomonas mendocina* Palleroni and *G. intraradices* or *G. mosseae* increased antioxidant enzyme activities (superoxide dismutase, catalase, and total peroxidase), phosphatase and nitrate reductase and solute accumulation in leaves of *Lactuca sativa* L. cv. Tafalla grown under severe drought stress (Kohler *et al*., 2008).

When *Gigaspora margarita* and *Acaulospora tuberculata* were inoculated with *Trichoderma asperellum* strain (PR11) increased plant height, root and shoot fresh weight and increased plant resistance against *Phytophthora megakarya* was recorded, increased synthesis of amino acids and phenolics in both healthy and infected plants indicated induced systemic response (Tchameni *et al*., 2011). Azcon, (1989) showed an interaction of PGPR with two AM fungi *G. mosseae* and *G. fasciculatum* in the roots of Lucerne (*Medicago sativa L.)* increased colonization of *G. mosseae* and *G. fasciculatum* in lucerne roots, indicating a role as mycorrhizal helper bacteria (MHB) (Pivato *et al*., 2008). These MHB induce and promote germination of AM fungal spores during the asymbiotic phase and promote hyphal growth in

presymbiotic phase (Pivato *et al.*, 2008), the effectiveness of these bacteria are reported to be AM fungal specific. Pivato *et al* (2008) found that the MHB *P. florescence* C7R12 increased the growth and association of *G. mosseae* BEG12 with medicinal plant *Medicago tarantula* but did not interact with *G. rosea* BEG 9. Interaction of these microorganisms can stimulate or suppress activity of pathogenic fungi and bacteria, making the mycorrhizosphere more microbiologically and biochemically active.

In the rhizosphere AM fungi interact with a variety of asymbiotic N_2 fixing bacteria, saprophytes, fungal and bacterial root pathogens, parasitic and pathogenic root nematodes. These fungi can act as bio-control agents against pathogens (Alam, 2000, Hamel, 1996). Their mode of control of pathogens involves several mechanisms such as increase in nutrient uptake and absorption, direct competition with the pathogen for infection sites, induced systemic resistance, productions of plant hormones, antibiotics, siderophores, and pathogenesis-related proteins. Several studies have shown that AM fungi also increased phenolic compounds, phenylpropanoid related enzymes and chalcone synthase all of which can trigger protection (Mummey *et al.*, 2009, Upadhyaya *et al.*, 2010). According to Jeffries *et al* (2003), the suppressive and bio-control effectiveness of AM fungi against disease incidence, pathogen development, and even disease severity is well documented. The bio-protective effect of these fungi is dependent on several environmental factors such as temperature, pH, soil moisture, and soil P contents. Other factors are host genotype, AM fungal species, agricultural practices and the degree of mycorrhization (Veirheilig *et al.*, 2008, Whipps, 2004). An important research study that demonstrated the bio-protective effect of AM fungi was conducted by Ozgonen *et al* (2010) who found that six mycorrhizal fungi namely *G. etunicatum, G. mosseae, G. clarum, G. caledonuim, G. fasciculatum,* and *Gi. margarita* decreased disease severity of stem rot caused *Sclerotium rolfsii* Sacc in peanut (*Arachis hypogaea* L.) by between 17.5 to 84.0%. A similar study by Tabin *et al* (2009) showed that *G. fasciculatum* reduced the percentage disease incidence of damping-off disease caused by *Pythium aphanidermatum* in *Aquilaria agallocha* seedlings. *G. intraradices* protected creeping bent-grass (*Agrostis palustris* cv Penncross) and velvet bent-grass (*Agrostis canina* cv Kingstown) against the take-all disease causative agent *Gaeumannomyces graminis* (Koske *et al.*, 1995).

1.4 SOIL

Soil is a finite and non-renewable resource regenerated through chemical and biological weathering of underlying rock (Doran and Safley, 1997, Nielsen and Winding, 2002). It is defined as a thin, unconsolidated, vertically differentiated portion of the Earth's surface, which is ubiquitous and often ignored despite its many important environmental and life-sustaining

functions. Again, it is important for agriculture, horticulture, natural environments, forests and sustainable development (Doran and Zeiss, 2000, Gugino *et al*., 2007 and 2009). The soil is made up by biological, physical and chemical properties that are usually related to soil health (Fig 1.3) (Abiwa and Wilmer, 2000, Gugino *et al*., 2009). These three properties are interconnected in sustaining soil health. Thus, chemical and physical properties are dependent on biological properties such as beneficial or detrimental microorganisms like viruses, bacteria, fungi, algae, protozoa, mites, nematodes, worms, ants, insects and insect larvae, small and larger animals which are all responsible for significant roles in the soil activities and food web (Clapperton, 2006, Doran and Safley, 1997). Microorganisms are responsible for several activities such as the breaking down of organic matter and cycling of N, C, P and other nutrients. They stabilize soil aggregate, structure, tilth, and productivity. However, manipulation of the soil through human-induced activities such as inappropriate agricultural management practices, deforestation, mining and natural disasters like earthquakes, volcanic eruptions and flooding results in changes in their diversity and population resulting in soil degradation (Chen *et al*., 2002). The term soil degradation is defined as the lowering and loss of soil functions, that poses a threat to agricultural production and terrestrial ecosystems (Bridges and van Baren, 1997, Chen *et al*., 2002). Certainly, any form of soil management can without a doubt negatively disturb soil resulting in poor soil health.

1.4.1 Soil health

Soil health is defined as the continued capacity of a living soil to fully function as a vital living system within natural or managed ecosystem regardless of land use boundaries, in order to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant animal and human health (Kinyangi, 2007, Gugino *et al*., 2009). However, this term is used interchangeably with soil quality. It is important to distinguish that, soil quality is related to soil function, whereas soil health presents the soil as a finite non-renewable and dynamic living system. The term soil health will be used here because it refers to soil microbiota and the vital roles they play in the functioning of living soils (Gugino *et al*., 2009, Nielsen and Winding, 2002). Soil health includes all three properties of soil and their relationship to building healthy soils (Fig 1.3). Therefore, proper monitoring and assessment of the effects of soil management needs to be implemented. The assessment and measurement of the effect of management practices on soil health especially biological properties is a big challenge and has mostly relied on the traditional physical and chemical properties of the soil. This approach ignores the importance of soil biological properties as a measure of the diversity and population of soil microorganisms.

1.4.2 Soil Biological Indicators

Biological indicators are defined as biological parameters that can be interpreted beyond the measured parameter itself (Avidano *et al.*, 2005). These indicators are measured by changes in the diversity, population, and activities of soil microorganisms (Benintende *et al.*, 2008, Nielsen and Winding, 2002). Soil health has been measured by biological indicators such as soil invertebrates (i.e. earthworms, millipedes, isopods, centipedes, and spiders), and protozoa, because they are most visible and do not require expensive methods to measure (Brown *et al.*, 2001, Schloter *et al.,* 2003). At the same time, microbial assays of bacteria and fungi are also used to indicate soil health because they play vital roles in soil and plant health. Microbial biomass and biodiversity can be measured with culture-dependent techniques such as physiological profiles and independent techniques making use of molecular analysis and phospholipids fatty acids (Table 1.2) (Arias *et al.*, 2005, Gugino *et al.*, 2009, Wakelin *et al.*, 2008). In addition, enzyme activity, bacterial diversity, richness, and density are used in combination appearing to be more revealing than a single parameter.

1.4.3 Soil Microorganisms

Microorganisms are important for the ecosystem functioning and measurement of soil. Thus, any changes in their functions, diversity, and population can serve as an indicator of the effect of agricultural management practice, because they are well adapted and sensitive to changes in their environment. They can be used as early warning signs or predictive tools in the soil health monitoring (Benintende *et al.*, 2008, Gugino *et al.*, 2009). The use of microbial indicators has being neglected due to lack of easy, reliable methods to measure, predict and quantify their behavior in the soil under different conditions. Recent studies in this field especially under agricultural management practices are focused on the assessment and the control of soil pathogens (Table 1.2) (Gugino *et al.*, 2009, Van Bruggen and Semenov, 2000). There is a need to thoroughly understand and gain knowledge of the different biological indicators of soil health, especially below ground. Likewise, the activity of mycorrhizal fungi is gaining more attention as it looks at few parameters like glomalin that is linked to soil chemical and physical properties as well as indicates activity. Several studies have used glomalin, AM fungal propagules and colonization percentage to determine soil health under different management practices (Bedini *et al.*, 2007, Schalamuk and Cabello, 2010).

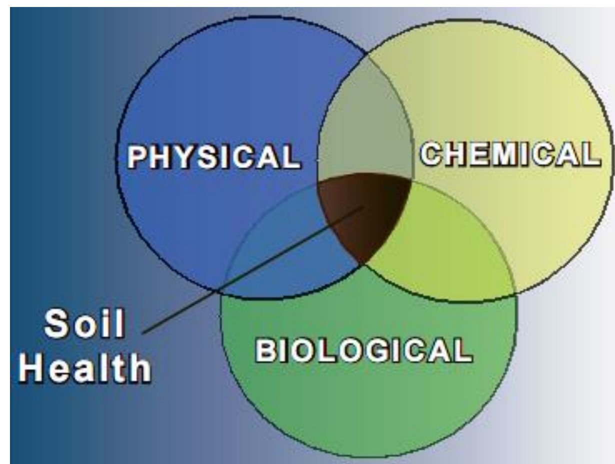

Figure 1-3 Schematic diagram showing soil health as the heart of physical, chemical and biological properties of soil (Gugino *et al*., 2009).

Table 1.2 Microbial indicators used in soil health monitoring (adapted from Arias *et al.*, 2005; Nielsen and Winding, 2000).

Soil ecosystem parameter	Microbial indicators	Current methods	Future methods
Biodiversity	Genetic diversity Functional diversity Marker lipids	PCR-DGGE BIOLOG ™ PLFA	T-RFLP Enzyme patterns, diversity of mRNA oligo-opiotrophs
C Cycling	Soil respiration Metabolic quotient (qCo2) Decomposition of organic matter Soil enzyme activity Methane Oxidation Methanotrophs	CO_2-production or O_2 consumption Litterbags $C_{resp}/C_{biomass}$ Enzyme assays Methane Measurement MPN, PLFA	Wood sticks FISH
N-cycling	N-mineralization Nitrification Denitrification N-fixation: Rhizobium N-fixation:cynobacteria	NH4+ accumulation NH4 + oxidation assay Acetylene inhibition assay Pot test MPN	Molecular methods
Microbial biomass	Microbial biomass: direct methods microbial biomass: indirect methods Microbial quotient Fungi Fungal-bacterial ratio Protozoa	Microscopy/PFLA CFI, CFE, SIR C_{mico}/C_{org} PLFA/Ergosterol PLFA MPN	MPN-PCR
Microbial activity	Bacterial DNA synthesis Bacterial protein synthesis RNA measurement Community growth physiology Bacteriophages	Thymidine incorporation Leucine incorporation CO_2-production or O_2-consumption	RT-PCR/FISH
Key species	Mycorrhiza Human pathogens Suppressive soil	Microscopy/Pot test Selective planting Pot test	Molecular methods Molecular/immuno logical methods
Bioavailability	Biosensors bacteria Plastid-containing bacteria Antibiotic-resistant bacteria incidence and expression of catabolic genes	Remediostm, Microtox ® Gel electrophoresis Selective growth Selective growth	New genetic constructions Molecular methods, Activity, Molecular/RNA measurements

1.5 Agricultural Management Practices

Agriculture is a term that refers to production of food and goods through farming and forestry. It is related to the intensive management of soil by practices such as long fallow periods, conventional tillage (also known as ploughing, cultivation, and digging of soil), and chemical application that were initially introduced to meet the always increasing demand for food supply. Practices employed to control and reduce pest, pathogens, and weeds has resulted in soil degradation by increased topsoil erosion by wind and water, and degradation of physical properties. Many of these practices have resulted in low levels of nutrients, groundwater pollution, a decline in water table, salinization, waterlogging, greenhouse gas emissions, acceleration of global warming, soil pollution and decline in crop production (Habte, 2006, Vanlauwe *et al.*, 2007). Most importantly they reduce microbial activity and diversity of below and above ground macro- and micro-organisms in the soil (Bendavid-Val *et al.*, 1997, Turk *et al.*, 2006). In South, Africa agriculture is still highly dependent on intensive conventional agricultural practices that are a major contributor to soil deterioration (VanBruggen and Semenov, 2000). Conventional agricultural soil management practices have been documented to have diverse effect on AM fungi because they create varying changes in the soil biological, chemical and physical properties that can influence the activity of AM fungi (Aggarwal *et al.*, 2010, Alguacil *et al.*, 2008). Therefore, changes in the relative proportions of AM fungal propagules appear to be a useful indication of the effect of these practices on soil health (Schalamuk *et al.*, 2006).

1.5.1 Conventional Management Practices

Previous studies show that conventional tillage breaks hyphal networks and dilutes AM fungal propagules resulting in reduced infectivity of these propagules, activity, host colonization and production of glomalin (Aggarwal *et al.*, 2010, Alguacil *et al.*, 2008, Castillo *et al.*, 2006). Soil preparation can result in destruction of hyphal networks that can alter the life cycle (Bethlenfalvay, 1992) and functionality of these fungi (Castillo *et al.*, 2006, Douds *et al.*, 1995). Intensive preparation of the soil redistributes propagules by mixing of topsoil effectively diluting the concentration of these propagules in the soil profile, resulting in reduced effectiveness (Douds *et al.*, 1995, Franke-Synder *et al.*, 2001, Galvez *et al.*, 2001, Honermeier, 2007). Therefore, changes in the number of AM fungal spores are widely used as bio-indicators or measures of the impact of tillage management practices on these fungi. From literature, the numbers of spores that can be recorded in agricultural soil can range from as low as one spore/g of soil to as high as 100 spores per g of dry soil depending on the type of soil, plant species and soil management practiced (Roldan *et al.*, 2007, Schalamuk and Cabello, 2010, Troeh and Loynachan, 2003). On the contrary, an even higher number of spores ranging from

20 to 150/ g of soil can be recorded in undisturbed soils, as compared to cultivated fields (Clapp *et al.*, 1995, Roldan *et al.*, 2007, Säle *et al.*, 2015). For example, a pilot study conducted in the Bophirima District in the Ganyesa sub-region of North West province of South Africa reported a low number of spores in cultivated soil compared to natural, grassland and pastures soil (Ingleby and Dirk, 2006). Bedini *et al.*, (2007) reported total numbers of spores ranging from 59 to 464 spores/ 100 g from cultivated dry soil and numbers of 401 to 4247 spores/ 100 g in undisturbed grassland soil. Changes in spore numbers can also be linked to AM fungal spore taxa.

Previous literature shows that intensive management of soil can select and favor certain taxa of AM fungi over others (Castillo *et al.*, 2006, Galvez *et al.*, 2001, Jansa *et al.*, 2003). AM fungal population and species composition is another approach used to indicate the effect of conventional management practices on soil health. In a recent study conducted by Schalamuk and Cabello (2010), they reported the presence of Acaulosporaceae, Gigasporaceae, and Glomeraceae in both no-till and conventional tillage, however upon establishment of trap cultures a high number of Glomeraceae were recovered such as *G. aggregatum*, *G. etunicatum*, *G. clarum* and *G. claroideum* indicating that the family is highly dependent on several soil factors and environmental conditions for their activity. In a study by Galvez *et al.*, (2001) they found that *G. etunicatum* spores were abundant under chisel disk tillage while *G. occultum* spores were more abundant under no-till, and *G. geosporum* spore were not affected by tillage. Therefore, the disturbance of soil at different levels can alter population composition. From the evidence provided above it is clear that conventional management practices do have an effect on the availability of AM propagules in the soil which impacts on their ability to form functional relationships with the host plant. This emphasizes the need for more research on sustainable agricultural practices such as conservation agriculture for better management of soil biological properties.

1.5.2 Conservation Management Practices

Conservation agriculture (CA) refers to the practice of minimal or no soil disturbance with maintenance of crop residue (mulch), soil moisture and soil aggregate stability in a given soil (Belder *et al.*, 2007). CA promotes reduction or no application of fertilizers and agrochemicals, reduced tillage (RT) or no-tillage (NT) of soil with cover cropping, crop rotation, and intercropping management practices. Their aim is to improve the chemical, physiological and biological properties, and to use the natural resources available for sustainable agricultural production more efficiently (Feng *et al.*, 2003). This approach was first introduced in the 1930’s in America and later adopted by Food Agriculture Organization (FAO) (Hobbs *et al.*, 2008). In Africa, CA started as early as the 1950s with the introduction of no-tillage practices.

The International Institute of Tropical Agriculture (IITA) of Nigeria and South Africa (SA) engaged in intensive NT practices during 1970s, and in 1983 Morocco followed (Mrabet, 2002). African countries such as Angola, Benin, Botswana, Ghana, Ivory Coast, Kenya, Malawi, Mozambique, Namibia, Niger, Nigeria, Senegal, Tanzania, Zambia, and Zimbabwe are now practicing CA on small-scale farms and research stations (Muok *et al*., 2009, Odhiambo and Ariga, 2001, Parsons, 2003, Tsubo *et al*., 2003). Adoption of CA as a soil management practice can help to rehabilitate, build and maintain soils for sustainable agriculture to meet the high demand of food supply. When compared with conventional agricultural practices the conservation approach is regarded as more sustainable because the focus is on building up and maintenance of healthy soils (Derpsch *et al*., 2010, Mathimaran *et al*., 2007). CA supports the growth, activity, and biodiversity of beneficial microorganisms such as actinomycetes, *Trichoderma* spp., and *Gliocladium* spp in the soil (Balota *et al*., 2004, Gil *et al*., 2008). For example, Zarea *et al*., (2011) study show that a mixed cropping system together with inoculation of AM fungi *G. mosseae* stimulated microbial activities, increased plant growth, N uptake, and nitrogenase activity of root nodules in forage legumes.

Previous studies have reported that the CA approach can reduce soil degradation (Diop, 1999), by promoting soil aggregates stability, reducing soil erosion, desertification and subsequently results in improved yield and productivity in agricultural soils. Overall these practices improve soil health and water quality, enhance soil nutrient contents and carbon sequestration, increase moisture content availability and control weeds, pest and root diseases (Abiwa and Wilmer, 2000, Baloyi *et al*., 2009, Dabney *et al*., 2001, Human, 2008, Karasawa *et al*., 2002, Moore *et al*., 1994, Nel and Loubser, 2004). The adoption of a CA approach is also more economically viable for farmers as there is a reduction in the use of expensive agrochemicals. The more favorable soil environment also stimulates the populations and activities of soil microorganism's especially beneficial AM fungi (Clapperton, 2006, Nielsen and Winding, 2000).

Current knowledge on the effect of cropping systems such as cover crops, crop rotation and intercropping on AM fungal activity is based on several successful studies. These practices all ensure a continued host presence for AM fungi. The absence of a suitable host reduces the number of viable AM fungal spores in the soil. During the asymbiotic phase germination in the absence of a suitable host will cease. Each successive germination attempt utilizes metabolically stored carbon until the spores are no longer active (Tommerup, 1984). Cover cropping defined as the use of crop such as legumes and cereal crops to establish cover for the soil have the potential of increasing AM fungal diversity and activity in the soil (Habte, 2000). In 1993, Douds *et al* found a higher population of AM fungi in low-input sustainable

agriculture with cover crops planted between cash crops. Millet (*Pennisetum americanum*), leek (*Allium* ampeloprasum var. *porrum*) sudangrass (*Sorghum bicolor* subsp. d*rummondii*) and milo (*S. bicolor*) cover crops are well known to improve AM fungal spore densities and maintain indigenous populations (Johnson and Pfleger, 1992; Kabir and Koide, 2002, Benitez *et al*., 2016). Sinegani and Sharif (2007) recorded 453 AM spores under *Allium cepa*, 255 spores from *Zea mays* and 115 spores from *Raphanus sativa* per 10 g of soil. The low numbers of spores under *R. sativa*, a member of the Brassicaceae, is understandable as plants in this family are generally non-mycorrhizal or are poorly colonised (Sinegani and Sharif, 2007) an important consideration when selecting cover crops.

Intercropping is defined as the growing of two or more different crops at the same time on a given piece of land (Mukhala *et al*., 1999), with cereal and legume crops being popular combinations. This practice promotes diversity of crops and can increase microbial diversity in the soil. Intercropping positively influence AM fungal populations provided suitable mycorrhizal host plants are selected. A study using *G. mosseae* inoculated sorghum (*Sorghum vulgare*) discovered that no mycorrhizal hyphal connections were established when cabbage (*Brassica oleraceae* L.) was used for intercropping (Ocampo, 1986). No-till or reduced tillage support Glomeraceae species better than the other families with few exceptions (Oehl *et al*., 2003, Jansa *et al*., 2003).

Crop rotation refers to a practice that involves growing of two or more different crops on the same piece of land one after another. This practice is the opposite of monoculture which is the continuous cropping of the same crop year after year. Crop rotation has being practiced for a long time in SA as a management tool to control soil-borne pathogens such as *Pseudocercosporella herpotrichoides, Fusarium spp., Gaeumannomyces graminis* and *Rhizoctonia cerealis* (Vilich, 1993). Strack *et al*, (2003) argues convincingly that sustainable agriculture should not exclude AM fungi, but it must enhance indigenous and inoculated populations as a measure of reducing the level of cultural stress. Again, the choice of a suitable mycorrhizal host plants is important as with cover crops and intercropping. Thus, monitoring and assessment of AM fungi provide a measure of soil disturbance and the status of soil health. Several studies have successfully used AM fungal activity and species composition to establish the effect of agricultural management practices on soil health worldwide (Aggarwal *et al*., 2010, Mathimaran *et al*., 2007). However, more still needs to be done especially in Africa and South Africa given that a high success rate of CA practices in many parts of the world is been reported with improvement in soil health and increased crop productivity (Honermeier, 2007, Johansson *et al,* 2004). Therefore, the effect of agricultural management

practices on the population and activity of AM fungi in the soil can be inter-connected to soil and plant health.

1.6 Motivation for the study

Over 80 % of land in South Africa is used for agriculture and only 11 % is arable (Mukhala *et al*., 1999). South African soil is characterised by low fertility, poor structure, low aggregate stability, reduced bulk density and soil biology. According to Derpsch (2007), the adoption of large-scale Conservation Agricultural (CA) management practices in South Africa is still confined to research stations and most of the data is not freely available for farmers (Derpsch, 2007). Current research on the effect of CA on soil biology in South Africa is focused on the reduction of soil-borne pathogens, nematodes, and bacteria with little focus on beneficial soil biology such as AM fungi. Changes to the management of soil should be measured especially now because the effect of CA practices on soil health is increasingly receiving attention worldwide including South Africa (Mills and Fey, 2003). Therefore, the lack of scientific data and information regarding the effect of agricultural management practices on soil biology under South African soil and environmental conditions prompted the need to investigate the potential use of AM fungi as biological indicators of soil health. Thus, this study is one of the few researches conducted in South Africa in order to determine the effect of agricultural practices on soil health. The main aim of this study was to assess changes in AM fungal activity through assessment of suitable parameters to measure soil health under conventional and conservation agricultural management practices in South Africa.

1.6.1 Hypothesis

This study hypothesis that Arbuscular mycorrhizal (AM) fungal soil propagules are good bio-indicator of soil health status under conventional and conservation agricultural management practices.

1.6.2 Objectives

The following objectives were formulated to assist in achieving the aim of the study:

(a) To assess AM fungal spores and propagule infectivity.
(b) To Identify indigenous AM fungal spore taxa in the soil.
(c) To quantify easily extractable glomalin (EEG) related soil protein in the soil.
(d) To assess AM fungal root colonization.

2. MATERIALS AND METHODS

2.1 Trial site historical description

The trail was established in 2007 under the ownership of Agricultural Research Council–Institute of Soil, Climate, and Water (ARC-ISCW). It was designed, established and maintained by researchers from ARC-ISCW. The experimental farm is situated at Zeekoegat, off the Moloto Road in Dinokeng South, Tshwane, Gauteng Province, South Africa (25º36’55” S and 28º18’56” E). This experimental farm belongs to the ARC-Animal Production Institute (ARC-API). Previously it was used for cultivating lucerne (*Medicago sativa* L.). Subsequently, five years before establishment of this study trial, the site was yearly tilled and left fallow. The soil at Zeekoegat consists mostly of deep red soils with moderately fine to medium blocky structure and clay texture. The soil form is Short lands, with underlying Gabbro. Concretions occur from 60 cm and it is more abundant on the south side of the trial (Fey, 2010).

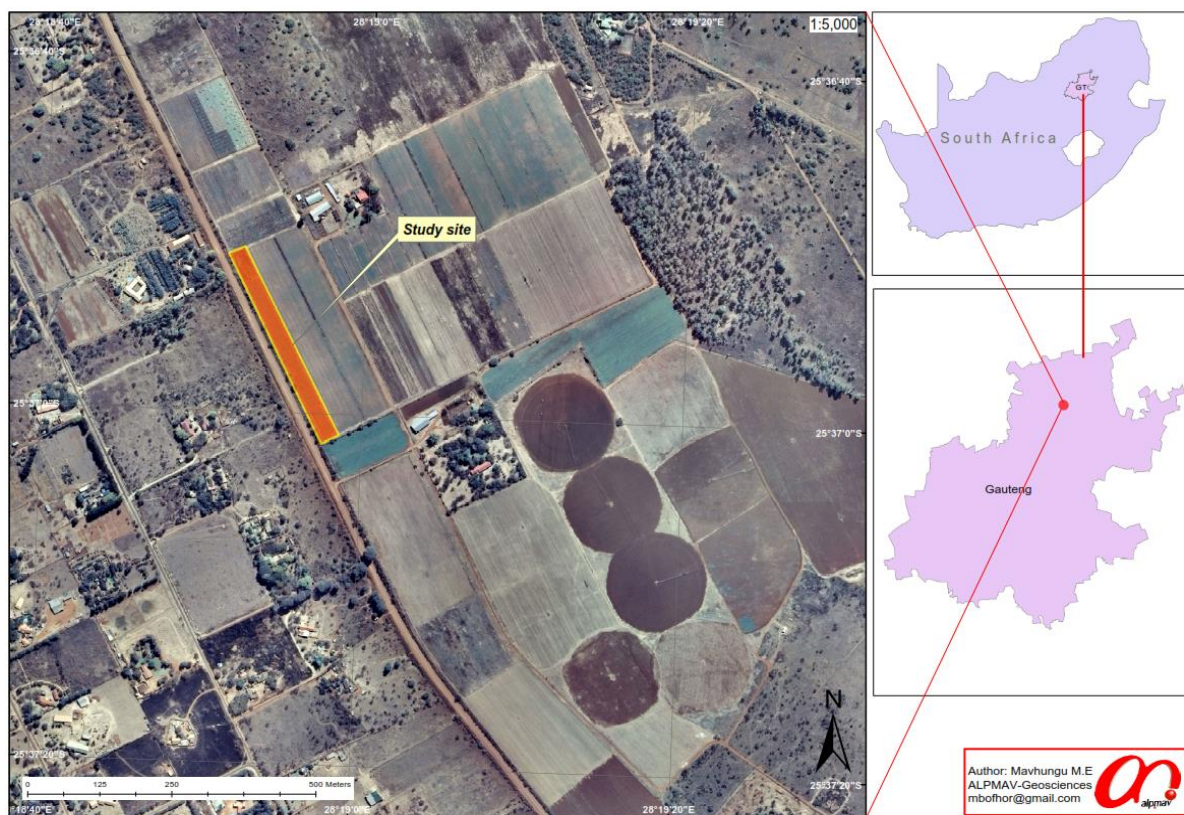

Figure 2.1 Zeekoegat study site in Gauteng South Africa.

2.2 Experimental design

The trail was a split-plot randomised complete block design (RCBD), replicated 3 times with each replicate split into 2 tillage system (whole plots) and each whole plot (conventional and reduced tillage) subdivided into 12 treatments (6 crop x 2 fertilizer) giving a total of 72 plots, (3 replicate blocks x 2 tillage x 2 fertilizer x 6 crop = 72 plots).

	Plot NO	**Treatments**		**Plot NO**	**Treatments**	
Block A Reduced tillage North	1	FH-MM		13	FH-MM	Block A Conventional Tillage
	2	FL-MC		14	FL-MC	
	3	FH-MS		15	FH-MS	
	4	FL-MS		16	FL-MS	
	5	FL-MM		17	FL-MM	
	6	FH-Mo		18	FH-Mo	
	7	FL-Mc		19	FL-Mc	
	8	FH-Mv		20	FH-Mv	
	9	FL-Mv		21	FL-Mv	
	10	FH-MC		22	FH-MC	
	11	FH-Mc		23	FH-Mc	
	12	FL-Mo		24	FL-Mo	
Block B Reduced tillage center	25	FL-Mo		37	FL-Mo	Block B Conventional Tillage
	26	FH-Mo		38	FH-Mo	
	27	FH-MC		39	FH-MC	
	28	FH-Mc		40	FH-Mc	
	29	FH-MM		41	FH-MM	
	30	FL-MC		42	FL-MC	
	31	FH-MS		43	FH-MS	
	32	FL-Mv		44	FL-Mv	
	33	FH-MM		45	FH-MM	
	34	FL-Mc		46	FL-Mc	
	35	FL-MS		47	FL-MS	
	36	FH-Mv		48	FH-Mv	
Block C Conventional Tillage	49	FL-MM		61	FL-MM	Block C Reduced tillage south
	50	FH-Mo		62	FH-Mo	
	51	FL-MC		63	FL-MC	
	52	FL-Mo		64	FL-Mo	
	53	FH-Mc		65	FH-Mc	
	54	FH-MC		66	FH-MC	
	55	FH-MS		67	FH-MS	
	56	FL-MS		68	FL-MS	
	57	FH-Mv		69	FH-Mv	
	58	FH-MM		70	FH-MM	
	59	FL-Mv		71	FL-Mv	
	60	FL-Mc		72	FL-Mc	

Figure 2.2 Layout of the Zeekoegat trail illustrating the split-plots of the randomised complete block design replicated three times with each replicate divided into two tillage systems. Treatments are abbreviated as FL = fertilizer low input; FH = fertilizer high input; MM = maize

monoculture; MS = maize-soybean rotation; MC = maize cowpea rotation; Mc = maize cowpea intercropping; Mo = maize oats intercropping; Mv = maize vetch intercropping.

Table 2.1 Sub-treatments within main conventional and reduced tillage split treatments.

Treatment Number	Fertilizer Input	Crops
1	Low input	Maize monoculture (FL-MM)
2	Low Input	Maize + cowpea (rotation) (FL-MC)
3	Low Input	Maize + soybean (rotation) (FL-MS)
4	Low Input	maize + cowpea (intercropping) (FL-Mc)
5	Low Input	Maize + oats (delayed intercropping) (FL-Mo)
6	Low Input	Maize +vetch (delayed intercropping) (FL-Mv)
7	High input	Maize monoculture (FH-MM)
8	High input	Maize + cowpea (rotation) (FH-MC)
9	High input	Maize + soybean (rotation) (FH-MS)
10	High input	maize + cowpea (intercropping) (FH-Mc)
11	High input	Maize + oats (delayed intercropping) (FH-Mo)
12	High input	Maize +vetch (delayed intercropping) (FH-Mv)

The crops used for this study were yellow maize (*Zea mays*) cv PAN 6P/110, cowpea (*Vigna unguiculata* cv Glenda), oats (*Avena sativa* cv 55H491), soybeans (*Glycine max*) cv PAN 854 (1412) and grazing vetch *(Vicia dasycarpa*) all obtained from PANNAR.

2.3 Soil preparation, fertilizer application and planting of trail per growing season studied

Figure 2.3 Soil preparations before planting under reduced tillage. A)-ARC-API staff opening rows with a tractor. B)-Opened rows in reduced tillage plots.

Figure 2.4 Labourers opening furrows with hoes for planting delayed intercrops. A)-Opening of furrows with hoes in conventional tillage plots. B)-Opening furrows in reduced tillage plots showing crop residue build-up on soil surface from previous season crop.

2.3.1 Soil preparation and planting

a) Soil preparation and planting for growing season 2008/2009

Conventional tillage plots were prepared by ploughing with a mouldboard plough tractor at 20 cm depth. Reduced tillage (RT) plots were not ploughed, soil preparation took place on the 17 & 18 November 2008 for growing season 2008/2009 using an open furrowing method (Fig. 2.3). The application of fertilizers for maize rows at low input was 100 kg of limestone ammonium nitrate (LAN) and 50 kg of potassium chloride (KCl). For high fertilizer input, 200 kg/ ha of LAN and 54 kg of N were applied. Under both reduced and conventional tillage Yellow maize cv PAN6/ 110 was planted by opening furrows with hoes and the furrows were closed completely. On the 20th of November 2008, the trail was sprayed with 2 % Roundup (active ingredients: Glyphosate) and Dual S Gold (active ingredient: S-Metholachior) at 1 L/ha. To control cutworm 400 ml/ha of the pesticide Cyperin (active ingredients: Cypermethrin (Pyrethroid) - 200 g/ℓ) was sprayed under both conventional and reduced tillage. On 8 December 2008, 350 ml/ ha of Cyperin was sprayed to control stalk borers. On 17 December 2008, intercropping and rotation plots were planted with cowpea cv Glenda. The delayed intercrop oats was planted on 25 February and vetch on 29 February 2009 at a density of 50 kg/ ha and vetch at a density of 30 kg/ ha (Fig. 2.3). Fertilizer application for legume rows was 100 kg/ ha of LAN and 50 kg/ ha of KCl for low input and 100 kg/ ha of LAN with 50 kg/ ha of KCl for high input. After planting the area was sprayed with 1.2 L/ ha of Dual S Gold and 3 L/ ha of Roundup herbicides to control emerging grass or weeds.

b) Soil preparation and planting for growing season 2009/2010

Planting for this growing season 2009/2010 started on 18 November 2009 for Block A only (Fig 2.2). On the 19 November 2009 planting continued for Block B and on 23 November 2009 for Block C (Fig 2.2). Planting for this season was delayed due to rain but each block was completed within a day, therefore variation within replicates was kept to a minimum. Cowpea intercrops were planted on 17 December 2009, 3 weeks after the main crops. The delayed intercrop oats were planted on 5 March 2010 and vetch on 9 March 2010. For this growing season application of fertilizers (LAN, P, and KCl), pesticides (Cyperin) and herbicides (Dual S Gold, Roundup) was the same as applications used for the previous growing season 2008/2009.

c) Soil preparation and planting for growing season 2010/2011

In this growing season 2010/2011, maize was planted on the 30 November 2010 and rotational legumes on the 21 December 2010. For the first time arbuscular mycorrhizal (AM) fungi inoculum obtained from Mycoroot ™ (Pty) Ltd, Grahamstown, South Africa was applied at a rate of 50 kg/ ha to all rows of the 72 plots and translated into 60 g of inoculant was equally spread over the row (Appendix C). The application of fertilizers was the same as previous growth season. The inoculum was applied after applying fertilizers and then the seeds.

d) Soil preparation and planting for growing season 2011/2012

In the fourth growing season, conventional treatments were ploughed with a mouldboard plough and then disked with a disk harrow on 28 November 2011. The reduced till plots were only slashed with hoes to open rows. Furrows were drawn and made manually by hand held hoe on both reduced and conventional tillage plots. Planting took place on 28 and 29 November 2011. Nitrogen fertilizer in the form of LAN was applied at 42 kg/ ha, with a follow-up fertilizer application of 28 kg N/ ha. Application for high fertilizer treatments (FH) was 108 g of LAN per row and low fertilizer treatments (FL) received half of FH. Phosphorus application was applied at 8 kg P/ ha (4 x 2). Supergrow (20.3%) was used as a P fertilizer and applied as follows 70 g per row for high fertilizer application (FH) and 35 g per row for low fertilizer application (FL). Potassium was not applied because resident levels in the soil were still high. Mycorrhizal inoculum was not reapplied.

Table 2.2 Crops and cropping system from 2008 to 2012 growing seasons.

Cropping system	Season 1 2008/2009	Season 2 2009/2013	Season 3 2010/2011	Season 4 2011/2012
1. Monoculture	Maize	Maize	Maize	Maize
2. Rotation	Maize	Cowpea	Maize	Cowpea
3. Rotation	Maize	Soybean	Maize	Soybean
4. Intercropping	Maize	Maize/cowpea	Maize	Maize/cowpea
5. Delayed intercropping	Maize	Maize/oats	Maize	Maize/oats
6. Delayed intercropping	Maize	Maize/vetch	Maize	Maize/vetch

2.4 Sampling

In all the four growing seasons soil and plant samples were collected in mid-February. During the first two growing seasons, five random samples were collected per plot. For the last two growing seasons, three samples were collected per plot to reduce soil disturbance within the plots. The samples were collected by removing the whole root system of plant (maize or legume) together with its surrounding soil respectively into brown paper bags (10kg) and immediately transported to a storage room at ARC-PPRI, Roodeplaat campus. Roots were removed, and soil samples were allowed to air dry for two weeks at 25ºC. Sub-sample of 250g of soil was collected for spore isolation, glomalin quantification and most probable number (MPN) from the main sample, these were stored in plastic bags in the 4ºC cold room until processing.

Immediately after collection of samples, plant samples were washed with tap water for 2-3 days. After washing the roots were placed in plastic bags and stored in a 4°C cold room. Subsamples of feeder roots for root colonisation assessment were collected and cut into approximately 1-2cm root pieces. The sub-samples were placed in McCartney 10ml bottles with 50% ethanol and stored at 25ºC.

2.5 Assessment of AM fungal activity in the soil

2.5.1 AM fungal spore's extraction and enumeration

A 100g of air-dried soil was measured from the stored 250g sub-sample and placed in a 2000ml beaker containing 1900ml of tap water. The suspension was agitated and then allowed to settle for 1-5 min. The suspension was decanted through a nest of sieves with mesh

sizes of 425µm, 250µm, 106µm, and 53µm. The contents of 425µm sieve were discarded and the residue on the remaining sieves was washed into 15ml Corning (430790-Labcon) centrifuge tubes. The spore suspension was centrifuged at 3000 rpm using a Hermile Z 320 bench top centrifuge for 5 min and the supernatants were discarded. The pellet was re-suspended in 60% (w/v) sucrose solution and centrifuged for 5 mins at 3000 rpm. The supernatant was decanted into a 45µm sieve and washed to remove sucrose from the spores (Brundrett, 2009, Douds and Millner, 1999, Sylvia, 1994, Daniel and Skipper, 1982).

The spore suspension was washed onto a 90 mm diameter filter chromatography paper disc (Chr#3) divided into 1cm grids in a Buchner funnel connected to a vacuum pump (Millipore XX 5522050). After drying the filter papers per sieve were transferred into a clean petri dish. Spore enumeration was conducted under a dissecting microscope (Nikon SMZ-10). The enumeration included dead and viable spores that were distinguished from one another by their appearance and light reflection under the dissecting microscope (Brundrett, 2009; Douds and Millner, 1999; Sylvia, 1994). Spore numbers per sample sieve were recorded and the total number of spores per plot was determined as representative of AM fungal spore population in 100 g of soil sample.

2.5.2 Most probable number (MPN) assay

According to Smith and Dickson (1991) with few modifications, the most probable number (MPN) of propagules indicating infectivity of indigenous AM fungi was determined by using 12 samples from 2008/2009 growth season. An additional 12 samples from 2010/2012 growth season were used as representatives of infectivity of AM fungi after inoculation. Soil samples were diluted tenfold with pasteurised sandy soil. The first dilution of 10^{-1} was made by mixing 200 g of sample with 1800 g of pasteurised soil in a plastic bag. From the first dilution, 200 g was taken and mixed with 1800 g of pasteurised soil to make the second dilution of 10^{-2}. This process was repeated two more time to achieve a 10^{-3} and 10^{-4} dilution. Sorghum (*S. vulgare*) seeds were used as trap plants and they were surface sterilized in 1% sodium hypochlorite for 20 mins and rinsed thoroughly with water. For each dilution, five seedling tray holes were used. The seedling holes were planted with three sorghum seed each. The seedling trays were kept in the tunnel for 4 weeks with minimum and maximum temperature of 25°C and 32°C respectively under natural light. The plant roots from each dilution were carefully removed, washed, stained and examined as described in section 2.1.6 for the presence or absence of AM fungal colonisation under a compound microscope (Nikon YS 100). A plus (+) was given every time mycorrhizal structures were observed in the entire root system.

Most probable number was calculated using an equation that is based on probability theory made from the number of root systems colonised with AM fungi each of the dilution series (Smith & Dickson, 1997). The P values (P1, P2, and P3) were obtained from the results. The first P value (P1) was the number of mycorrhizal plants in the least concentrated dilution that had the highest colonisation. The number of plants that had the second highest mycorrhizal colonisation was P2 followed by the third highest (P3). Using the probability table the row with which P1 and P2 correspond to P3 was taken as the most probable number in that sample. The value was then multiplied by the dilution to give MPN per 100 g of soil (Smith and Dickson, 1997).

2.5.3 Identification of AM fungal spore taxa

Spores were directly mounted on microscope slides in PVLG, Melzers plus PVLG reagents (Appendix A) (1:1 v/ v) and water. The spores were identified by morphological descriptive characteristics such as colour, size, attachment, spore walls and germinating structures to determine genus (Khade, 2011, Koske and Walker, 1986, Thapar and Khan, 1973, Trappe, 1982, Walker and Sanders, 1986). Descriptions were compared to those given on the INVAM website (http://invam.caf.wvu.edu).

2.5.4 Glomalin (EEG) extraction and quantification

The glycoprotein, glomalin was extracted from soil samples collected. Easily extractable glomalin (EEG) was extracted from soil using a modified method of Wright and Upadhyaya (1996, 1998) described by Janos *et al.*, (2008). EEG in 1g of soil was measured and placed in 15ml centrifuge tubes. It was suspended in 20mM sodium tri-citrate (pH 7.0) solution and then extracted in an autoclave at 121°C for 45 mins. After autoclaving, the tubes were immediately centrifuged at 5000 rpm for 15 mins. The supernatants were transferred into 2 ml eppendorf tubes and stored at 4°C for quantification (Janos *et al.*, 2008).

The concentration of EEG (mg/g) was quantified according to the Bradford assay and bovine serum albumin (BSA) from Fermentas (# R1281) was used for the standards. BSA standard solutions with concentrations from 1.25-5.00mg/ml were used. Duplicates of 5µl of BSA standards, blank (water) as negative control and test samples were pipetted into 96 well micro-titre plates. A volume of 250µl of Bradford Coomassie brilliant blue dye (Bio-Rad) was added to each well and left at 25°C for 10-15 mins to allow for colour development. Absorbance was measured using a spectrophotometer (SpectraMax Plus 384) at 595nm. Absorbance was corrected for the blank and the values of the standards readings were calculated and plotted

against their concentration using a scatter graph with linear regression values and equation (Appendix B). The regression equation of $y = ax + b$ from a standard curve was used to determine samples concentration (x) which is the unknown concentration, values were corrected for amount of soil and extractant used.

2.6 Root analyses for AM fungal structures

2.6.1 Root staining for mycorrhizal colonization

Fixed roots (as described in section 2.4) were washed with distilled water (dH_2O). After washing the roots were covered with 5% (w/v) potassium hydroxide (KOH) solution and were incubated in a 90°C water bath (Gallenkamp) for 45 mins. Potassium hydroxide was discarded, and the roots were washed with dH_2O. Subsequently, the roots were bleached with freshly prepared alkaline hydrogen peroxide (H_2O_2) solution (Appendix A) for 30 mins or until roots turned white or clear. The solution was discarded, and the roots were again washed with dH_2O. Furthermore, the roots were acidified overnight in a 0.1M HCl solution (Appendix A). The HCl was discarded and the roots were covered with lactoglycerol solution containing 0.05% (w/v) trypan blue as a stain (Appendix A) (Koske and Gemma, 1989; Kormanik and McGraw, 1982) and incubated for 45 mins in a 90 °C water bath. The staining solution was decanted, and the roots were destained in clear lactoglycerol overnight (Appendix A). After two days root sections were mounted on slides in polyvinyl (PVLG) mountant for microscopic examination (Brundrett, 2009).

2.6.2 Percentage root colonization assessment

Stained root pieces were further analysed for percentage root colonization using a modified slide intercept method (Daniels and Skipper, 1982). Ten root pieces of 1-2 cm length were squashed on a slide after covering with coverslip. The roots were analysed for AM fungal structures (arbuscular, vesicles and intercellular hyphae) using of a compound microscope (Nikon Optiphot) at 40 x magnification. A minimum of 100 fields of views were examined. The presence of AM fungal structures inside the root pieces was recorded and total number of root pieces colonised was calculated (Douds and Millner, 1999; Kormanik and McGraw, 1982) and expressed as percentage root colonised.

Quantification was performed using the following formula: (McGonigle, 1990).

$$\text{Percentage AM fungal colonization (\%)} = \frac{\text{Root pieces colonized}}{\text{Total number of root pieces observed}} \text{ X } 100$$

2.7 Statistical Analysis

All data collected was subjected to an appropriate analysis of variance (ANOVA) using the SAS/STAT statistical software (SAS, 1999). The standardized residuals were tested for deviations from normality using Shapiro-Wilks test (Shapiro and Wilk, 1965). In cases were deviations from normality was evident because of skewness, outliers were removed until residuals were normal or symmetric distributed (Glass *et al*., 1972). For significant source effects, the Student's t-LSD (least significant difference) were calculated at 5% level of significance (Snedecor & Cochran, 1967) to compare means. In order to group the treatment combination in similar groups, a multivariate clustering (AHC = Agglomerative hierarchical clustering) procedure was performed, specifying the dissimilarity as Euclidean distance using Ward's method.

3. RESULTS

3.1 AM Fungal Spores

Despite the low numbers of indigenous (AM) fungal spores recovered in the first two growing seasons, some significant differences were recorded between treatments plots (Table 3.1). The mean numbers of spores in 100 g of dry soil were low for the first two growing seasons 1 and 2 with grand mean of 19 and 33, respectively (Table 3.1). These low numbers of spores prompted the need to inoculate with an AM fungal inoculant (Mycoroot SuperGro) (Appendix C) at the time of planting in the third growing season (2010/2011). Field inoculation significantly increased by more than 3-fold the number of AM fungal spores enumerated which was assessed 8 weeks after inoculum application (Table 3.1). This increase was maintained above 100 spores/g of soil in the fourth growing season without further inoculation, giving a grand mean of 118 and 133, in growing season 3 and 4 respectively (Table 3.1). Inoculation increased spores' numbers in all treatment plots; therefore, had a positive effect on AM fungal spore numbers (Table 3.1).

Initially, AM fungal spore numbers were low but increased under conventional tillage overall growing seasons as compared to reduced tillage (Table 3.1). These spore numbers also increased under fertilizer high input overall growing seasons as compared to fertilizer low input. They also increased consistently in maize monoculture (MM), maize cowpea (Mc) intercropping, and maize vetch (Mv) delayed intercropping overall growing seasons as compared to maize soybean (MS) rotation, maize cowpea (MC) rotation and maize oats (Mo) delayed intercropping (Table 3.1).

According to the analysis of variance, tillage coupled with fertilizer input had a significant effect ($P < 0.01$) on AM fungal spore numbers in growing season 1. The unprotected LSD analysis show that AM fungal spore numbers increased under conventional tillage coupled with fertilizer high input as compared to reduced tillage coupled with fertilizer high input with 39 and 37 spores / 100 g of dry soil respectively. Furthermore, ANOVA shows that tillage with fertilizer input and cropping systems had a significant effect ($P < 0.01$) on AM fungal spore numbers in growing season 1. The unprotected LSD analysis shows that AM fungal spore numbers increased under reduced tillage, fertilizer high input with maize cowpea rotation and maize vetch intercropping.

Fertilizer application had a significant effect ($P<0.05$) on AM fungal spore numbers in growing season 2 only. The AM fungal spore numbers increased under fertilizer high input as compared to fertilizer low input. Overall, fertilizer high input appears to be the main driver

impacting on AM fungal spore activity (Table 3.1 & 3.2) particularly in season 1 and 2 representing the indigenous spore population in the soil.

Table 3.1 Mean number of AM fungal spores in treatment plots/100 g of dry soil for four growing seasons.

Tillage	Fertilizer	Cropping system	Growing seasons							
			2008/2009		2009/2010		2010/2011#		2011/2012	
Grand Mean			19		33		118		133	
Tillage										
Conventional Tillage			17	a	38	a	119	a	159	a
Reduced Tillage			21	a	28	a	118	a	108	a
		P value	*0.46*		*0.14*		*0.95*		*0.46*	
Fertilizer										
High Fertilizer			20	a	37	a	123	a	143	a
Low Fertilizer			18	a	29	b	114	a	124	a
		P value	*0.59*		*0.04*		*0.57*		*0.37*	
Cropping system										
MM			16	b	28	a	82	b	163	ab
MS			18	ab	37	a	121	ab	93	b
MC			19	ab	30	a	164	ab	121	ab
Mc			18	ab	40	a	96	b	126	ab
Mo			20	ab	26	a	121	ab	113	ab
Mv			24	a	36	a	130	ab	185	ab
		LSD (P = 0.05)	*9*		*15*		*49*		*73*	
Tillage X Fertilizer										
Conventional Tillage										
	High Fertilizer		15	b	41	a	115	a	168	a
	Low Fertilizer		19	ab	35	a	123	a	149	ab
Reduced Tillage										
	High Fertilizer		25	a	33	ab	133	a	117	ab
	Low Fertilizer		17	b	22	b	104	a	99	b
		LSD (P = 0.05)	*7*		*12*		*40*		*59*	
Tillage X Cropping System										
Conventional Tillage										
	MM		13	b	28	bc	67	d	206	ab
	MS		16	ab	45	ab	103	bcd	116	bc
	MC		13	b	34	ab	157	ab	145	abc
	Mc		22	ab	53	ab	106	abcd	145	abc
	Mo		16	ab	22	c	119	abcd	12	bc
	Mv		22	ab	46	ab	159	ab	228	a
Reduced Tillage										
	MM		18	ab	28	bc	96	bcd	119	bc
	MS		19	ab	29	bc	140	abc	70	c
	MC		27	a	26	bc	173	a	97	c
	Mc		14	b	27	bc	87	cd	108	bc
	Mo		24	a	30	bc	119	abcd	115	bc
	Mv		28	a	26	bc	102	bcd	141	abc
		LSD (P = 0.05)	*12*		*21*		*69*		*103*	

#2011/2012 was inoculated with mycorrhizal inoculum. Abbreviations: MM = maize monoculture; MS = maize soybean rotation; MC = maize cowpea rotation; Mc = maize cowpea intercropping; Mo = maize oats intercropping; Mv = maize vetch intercropping. LSD

= Least significant difference within season. Means indicated with the same letters were not significantly different.

Table 3.1 Continued

Tillage	Fertilizer	Cropping systems	Growing seasons							
			2008/2009		2009/2010		2010/2011#		2011/2012	
Fertilizer X Cropping System										
	High Fertilizer									
		MM	16	ab	29	bc	81	b	180	ab
		MS	23	ab	46	ab	115	b	92	b
		MC	22	ab	27	bc	209	a	118	b
		Mc	19	ab	58	ab	101	b	88	b
		Mo	17	ab	26	bc	112	b	148	ab
		Mv	22	ab	38	abc	136	b	231	a
	Low Fertilizer									
		MM	15	ab	27	bc	82	b	145	ab
		MS	13	bcde	28	bc	127	b	94	b
		MC	15	ab	34	bc	127	b	124	b
		Mc	17	ab	22	c	92	b	165	ab
		Mo	24	ab	26	bc	130	b	79	b
		Mv	26	a	35	bc	124	b	138	ab
		LSD *(P = 0.05)*	12		21		69		103	
Tillage X Fertilizer X Cropping system										
Conventional Tillage										
	High Fertilizer									
		MM	13	cde	28	bcde	70	bc	223	ab
		MS	14	cde	57	ab	105	bc	107	bc
		MC	9	e	23	de	164	b	146	abc
		Mc	27	abcd	78	a	104	bc	108	bc
		Mo	14	cde	21	de	80	bc	136	bc
		Mv	11	cde	40	bcde	165	b	289	a
	Low Fertilizer									
		MM	14	cde	28	bcde	64	c	188	abc
		MS	18	bcde	33	bcde	101	bc	124	bc
		MC	17	bcde	46	bcde	150	bc	143	bc
		Mc	17	bcde	28	cde	107	bc	182	abc
		Mo	19	bcde	23	de	166	bc	88	bc
		Mv	32	ab	53	abc	152	bc	167	bc
Reduced Tillage										
	High Fertilizer									
		MM	19	bcde	29	bcde	93	bc	136	bc
		MS	31	ab	35	bcde	126	bc	76	c
		MC	42	a	31	bcde	277	a	90	bc
		Mc	11	de	38	bcde	97	bc	68	c
		Mo	20	bcde	31	bcde	143	bc	160	abc
		Mv	39	a	36	bcde	107	bc	173	abc
	Low Fertilizer									
		MM	17	bcde	26	cde	100	bc	103	bc
		MS	7	e	23	de	154	bc	64	c
		MC	11	cde	22	de	104	bc	104	bc
		Mc	17	bcde	16	e	78	bc	147	abc
		Mo	29	abc	29	bcde	94	bc	70	c
		Mv	20	bcde	17	de	96	bc	109	bc
		LSD *(P = 0.05)*	*17*		*29*		*98*		*145*	

#2011/2012 was inoculated with mycorrhizal inoculum. Abbreviations: MM = maize monoculture; MS = maize soybean rotation; MC = maize cowpea rotation; Mc = maize cowpea intercropping; Mo = maize oats intercropping; Mv = maize vetch intercropping. LSD = Least

significant difference within season. Means indicated with the same letters were not significantly different.

Table 3.2 Analysis of variance of arbuscular mycorrhizal fungal spores/100g soil over four growing seasons.

		Growing seasons							
		2008/ 2009		2009/2010		2010/2011		2011/2012	
Source of Variation	*df*	Mean Square	P value	Mean Square	P value	Mean Square	P value	Mean Square	P value
Block stratum	2	70.33	NS	3052.22	NS	210433.95	NS	36235.06	NS
Tillage	1	279.67	NS	1972.97	NS	58.76	NS	45317.22	NS
Error (a)	2	339.51		337.82		11470.75		53759.71	
Fertilizer application	1	30.10	NS	1352.87	*	1161.25	NS	6353.15	NS
Tillage X Fertilizer application	1	688.45	**	118.84	NS	5118.31	NS	13.35	NS
Cropping System	5	113.09	NS	379.55	NS	8634.34	NS	13759.78	NS
Tillage X Cropping systems	5	126.98	NS	478.38	NS	3539.40	NS	3409.43	NS
Fertilizer application X Cropping systems	5	118.64	NS	748.12	NS	3160.71	NS	11102.20	NS
Tillage x Fertilizer application X Cropping systems	5	382.44	**	422.96	NS	4058.50	NS	954.56	NS
Error (b)	41	104.09		316.74		3454.12		7773.70	
Total	68	2253.3		9180.5		251090.1		178678.1	

2010/2011 was inoculated with mycorrhiza, NS = not significant, * = P ≤ 0.05, ** = P ≤ 0.01.

3.2 Most probable number (MPN)

Despite the low AM fungal spore numbers in the first two growing seasons, infectivity was recorded under glasshouse-controlled environment. According to the root assessment of host crop used in the trap culture, MPN increased with inoculation under both tillage treatments. Therefore, AM fungal propagules Infectivity increased under both conventional and reduced tillage from 40/2 propagules/ 100g of soil before to 610/45 propagules/ 100g of soil after inoculation respectively (Table 3.3). The MPN results also shows increased infectivity under both fertilizer input coupled with maize monoculture (MM). Infectivity was negatively affected by maize vetch under both tillage and fertilizer inputs. Therefore, maize vetch (Mv) delayed intercropping had a negative effect on AM fungal propagules. MM cropping system positively increased MPN under both tillage and fertilizer application treatments (Table 3.3). Therefore, MPN values were highly influenced by proceeding and succeeding field host crop.

Table 3.3 Most probable number (MPN) infectivity tests results of propagules per 100g of composite soil before and after inoculation with Mycoroot inoculum.

	MPN/100 g of dry soil	
Treatments	Before	After
Conventional tillage		
FHMM	40	610
FLMM	1.8	6.8
FLMS	2	9.2
FHMc	4	4
FHMv	140	45
Average	37.6	135
Reduced tillage		
FHMM	2	45
FLMM	20	110
FLMS	7.8	110
FHMc	11	20
FHMv	170	40
Average	42.2	65

FL = fertilizer low input, FH = fertilizer high input; MM = maize monoculture, MS = maize soybean rotation, MC = Maize cowpea rotation, Mc = maize cowpea intercropping, and Mv = Maize vetch intercropping.

3.3 Identification of AM fungal taxa

Identification of resident, indigenous background spores to species level was difficult due to the low number of spores extracted from the study site. Using only morphological features from the limited number of spores obtained tentative identifications to genus level was made. These identifications were based on background populations before application of the inoculum. Spore taxa observed were similar for both low and high fertilizer application, cropping systems and both reduced and conventional tillage, bearing in mind the low numbers of spores available for examination. The spores were identified to be from the following genera *Glomus, Gigaspora* and *Scutellospora* and descriptions are provided below.

3.3.1 Description of *Scutellospora* morphotype

Spores belonging to the *Scutellospora* genus were identified as white to yellow in colour, globose to sub-globose with an average diameter of 100-200 µm (Fig 3.1 A). The spores had bulbous subtending hyphae also referred as a sporogenous cell (sc) (Fig 3.1 B, C). The walls of the spores had a plate-like "germination shield" (gs) on the surface of the innermost flexible inner wall (Fig 3.1 A, D). The germination shield stained yellow to brownish in colour and 172 µm in size with small compartments, including 10 µm multiple germ tubes and a 10 µm germinal whole (Fig 31. A, D). Spore wall consisted of outer and inner, flexible layers that could not be properly distinguished (Fig 3.1 A-D – L1, L2).

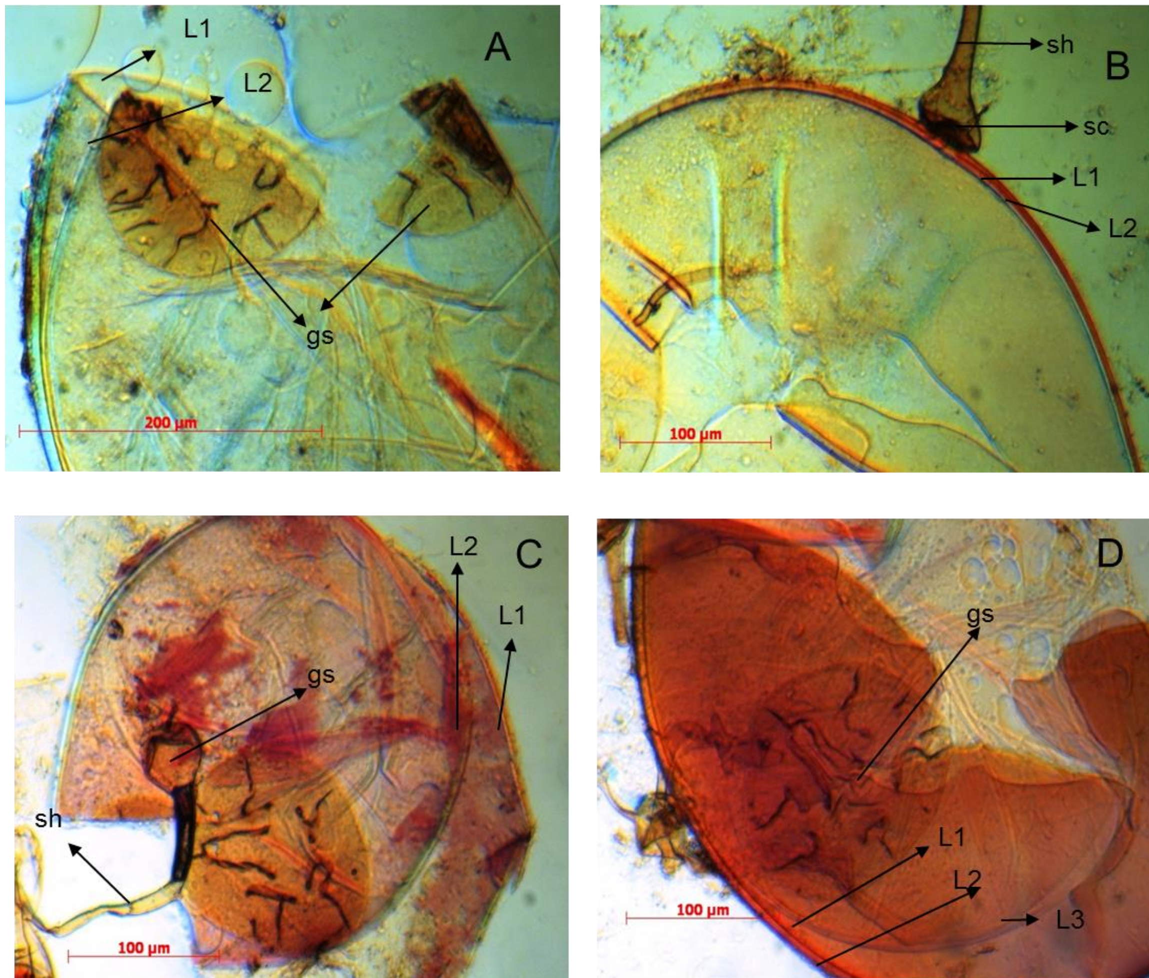

Figure 3.1 Spores of *Scutellospora* from Zeekoegat soil samples. A)-Brown yellow spore crushed in PVLG showing yellow-brown germinating shield (gs), outer wall layer (L1), inner wall layer (L2). B and C)-Yellowish brown spore in PVLG with defined outer layer and a bulbous sporogenous cell (sc). Spore patchily stained with meltzer's reagent, subtending hyphae (sh), germinating shield (gs), wall layer (L1 and L2). D)-A purple-pink stained spore in Meltzer's reagent and PVLG with germinating shield (gs), wall layer (L1, L2).

3.3.2 Description of *Gigaspora* morphotype

The genus *Gigaspora* was identified from spores that were globose to sub-globose in shape, varying in size from 250-448 µm with a mean of 349 µm in diameter (Fig 3.2 A). Spores stained pale yellow subtending hyphae (Fig 3.2 E - sh) that was 20 µm in diameter at its widest point (Fig 3.2 E –sc). Spores had 2 distinct spore walls layers (Fig 3.2 D, F – L1, L2) that stained pale yellow in Meltzer's stain.

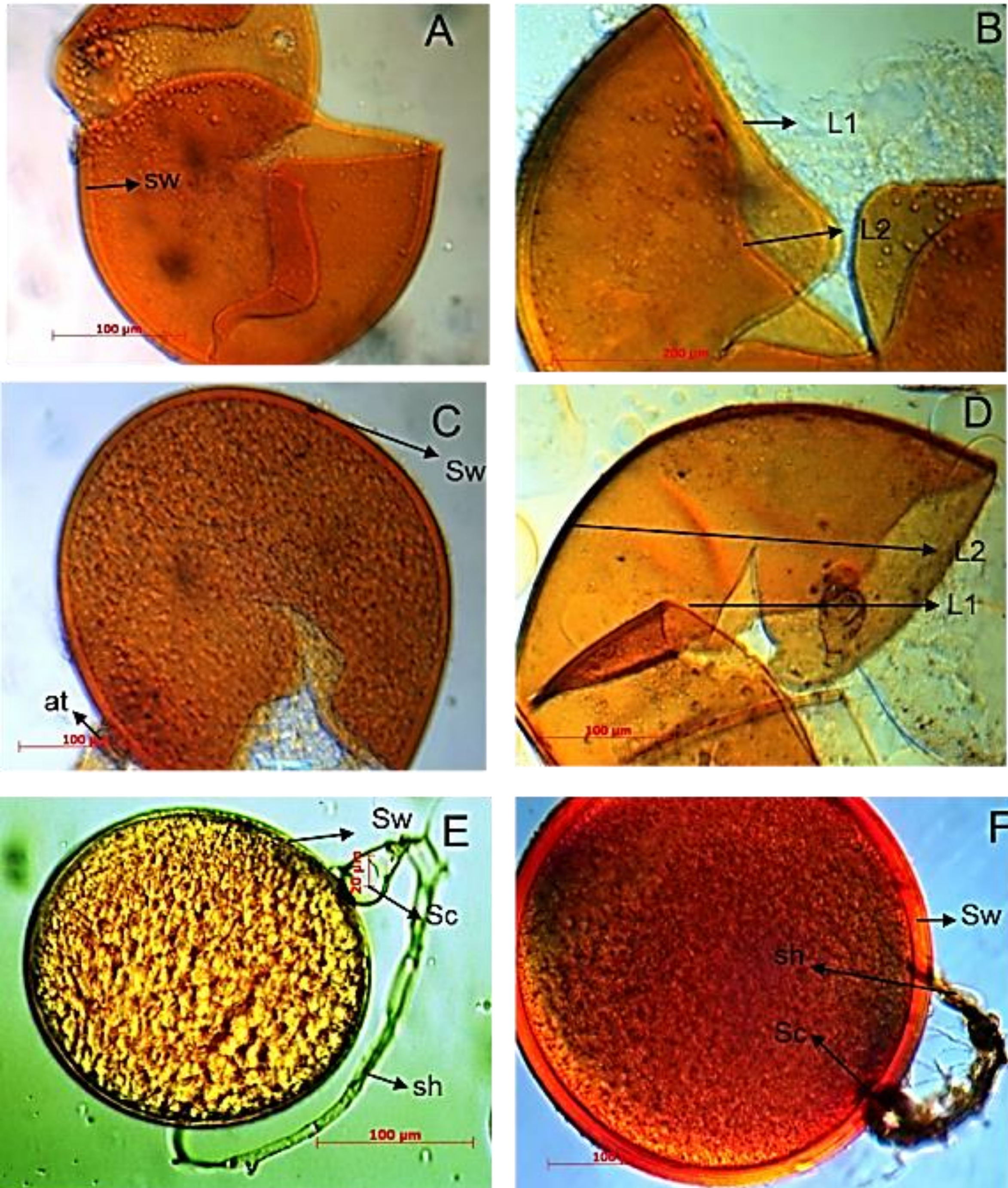

Figure 3.2 Spores of *Gigaspora* from Zeekoegat soil samples. A)-Brownish yellow spore stained in melzers reagent and PVLG showing spore wall (sw) after crushing. B)-Spore in PVLG showing spore wall layers (L1 and L2). C) -Spore showing spore wall (sw) and attachment (at). D)-Brown spore crushed in PVLG with layer one and two (L1 and L2). E)-Yellow spore with sw-spore wall, bulbous sporogenous cell (sc) with funnels shaped connectible and subtending hyphae (sh). F)-Spore red in meltzer's stain with spore wall (sw), bulbous attachment (sc) and subtending hyphae (sh).

3.3.3 Description of *Glomus* morphotype

The *Glomus* spores were identified as compact cluster of spores joined together by sporogenous hyphae. The spores were globose in shape and stained pale yellow in Meltzer's stain (Fig 3.3 A). Hyphal attachments stained hyaline to pale yellow (Fig 3.3 A, B). Spores had a single wall layer possibly surrounded by an outer covering. The subtending hyphae were cylindrical in shape (Fig 3.3 B - h).

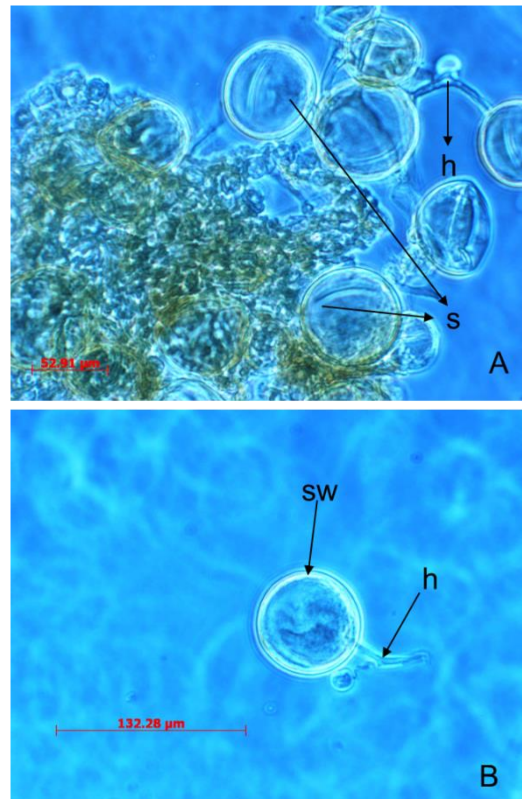

Figure 3.3 *Glomus* spores from Zeekoegat soil samples. A)-Spore with hyphal attachment (h) of aggregate spores (s). B)-Spores showing spore wall (sw) a single spore**.**

3.4 Glomalin (EEG) quantification

The concentration of recently deposited easily extractable glomalin (EEG) was low (< 2.5 mg/g soil) for all growing seasons with values ranging from 1.64 -2.57 mg/g soil (Table 3.4). The concentration of EEG increased with the application of inoculum, but this was not significant. The analysis of variance shows that fertilizer input and cropping systems had significant effect ($P < 0.01$) on EEG concentration in growing season 2 only (Table 3.5). This was supported by the unprotected LSD analysis shows that EEG concentration increased under both fertilizer regimes regardless of tillage treatment. The analysis shows that EEG concentration varied between cropping systems regardless of fertilizer input. Therefore, EEG increased under

fertilizer low input with maize monoculture (MM), maize soybean rotation (MS), Maize oats (Mo) and maize vetch (Mv) delayed intercropping. The concentration decreased under fertilizer low input with maize cowpea (Mc and MC) intercropping and rotation (Table 3.4). Furthermore, EEG increased under MS, Mc, MC, and Mo and decreased under MM and Mv. Therefore, the concentration of EEG was evidently influenced by both crop type and fertilizer input.

Table 3.4 Unprotected LSD mean values of EEG concentration in mg/g soil for four growing seasons.

Tillage	Fertilizer	Cropping system	Growing seasons			
			2008/2009	2009/2010	2010/2011[#]	2011/2012
Grand Mean			1.85	1.72	2.53	1.92
Tillage						
Conventional Tillage			1.89 a	1.69 a	2.51 a	1.94 a
Reduced Tillage			1.81 a	1.75 a	2.55 a	1.91 a
		P value	*0.35*	*0.50*	*0.13*	*0.76*
Fertilizer						
High Fertilizer			1.86 a	1.73 a	2.53 a	1.93 a
Low Fertilizer			1.84 a	1.72 a	2.52 a	1.92 a
		P value	*0.81*	*0.64*	*0.44*	*0.9*
Cropping system						
MM			1.83 a	1.70 b	2.53 a	2.07 a
MS			1.86 a	1.77 a	2.53 a	1.89 a
MC			1.89 a	1.70 b	2.53 a	1.89 a
Mc			1.88 a	1.71 ab	2.54 a	1.91 a
Mo			1.81 a	1.74 ab	2.54 a	1.84 a
Mv			1.85 a	1.71 ab	2.53 a	1.93 a
		LSD $_{(P = 0.05)}$	*0.10*	*0.06*	*0.03*	*0.32*
Tillage X Fertilizer						
Conventional						
	High Fertilizer		1.89 a	1.69 b	2.52 b	1.89 a
	Low Fertilizer		1.89 a	1.68 b	2.52 b	1.99 a
Reduced Tillage						
	High Fertilizer		1.82 a	1.76 a	2.55 a	1.96 a
	Low Fertilizer		1.80 a	1.75 a	2.54 a	1.85 a
		LSD $_{(P = 0.05)}$	*0.08*	*0.04*	0.02	*0.26*
Tillage X Cropping System						
Conventional tillage						
	MM		1.89 ab	1.71 c	2.51 bc	2.01 a
	MS		1.94 a	1.72 bc	2.52 abc	1.85 a
	MC		1.94 a	1.68 c	2.51 bc	1.85 a
	Mc		1.87 ab	1.68 c	2.52 abc	1.94 a
	Mo		1.91 ab	1.69 c	2.53 abc	1.92 a
	Mv		1.81 abc	1.67 c	2.49 c	2.06 a
Reduced tillage						
	MM		1.77 bc	1.69 c	2.54 ab	2.12 a
	MS		1.79 bc	1.81 a	2.54 ab	1.94 a
	MC		1.84 abc	1.71 bc	2.54 ab	1.94 a
	Mc		1.89 ab	1.75 abc	2.55 a	1.87 a
	Mo		1.72 c	1.80 ab	2.54 ab	1.79 a
	Mv		1.88 ab	1.74 abc	2.55 a	1.76 a
		LSD $_{(P = 0.05)}$	0.14	0.09	0.04	0.45

[#]2011/2012 was inoculated with mycorrhiza, Abbreviation: MM = maize monoculture; MS = maize soybean rotation; MC = maize cowpea rotation; Mc = maize cowpea intercropping; Mo = maize oats intercropping; Mv = maize vetch intercropping. LSD = Least significant difference within season with P value = 0.05.

Table 3.4 Continued:

Tillage	Fertilizer	Cropping system	Growth seasons							
			2008/2009		2009/2010		2010/2011#		2011/2012	
Fertilizer X Cropping System										
	High Fertilizer									
		MM	1.85	a	1.68	bc	2.51	a	2.06	a
		MS	1.91	a	1.78	abc	2.54	a	1.83	a
		MC	1.86	a	1.74	abc	2.54	a	2.00	a
		Mc	1.89	a	1.77	abc	2.55	a	1.98	a
		Mo	1.79	a	1.73	abc	2.54	a	1.68	a
		Mv	1.84	a	1.66	c	2.53	a	2.01	a
	Low Fertilizer									
		MM	1.81	a	1.72	abc	2.54	a	2.08	a
		MS	1.81	a	1.76	abc	2.53	a	1.96	a
		MC	1.92	a	1.67	c	2.52	a	1.79	a
		Mc	1.86	a	1.66	c	2.53	a	1.83	a
		Mo	1.84	a	1.75	ab	2.54	a	1.99	a
		Mv	1.86	a	1.76	ab	2.52	a	1.84	a
		LSD $_{(P = 0.05)}$	0.14		0.09		0.04		0.45	
Tillage X Fertilizer X Cropping system										
Conventional Tillage										
	High Fertilizer									
		MM	1.84	abc	1.71	bcde	2.49	abc	1.93	a
		MS	1.92	a	1.71	bcde	2.51	abc	1.88	a
		MC	1.94	a	1.70	bcde	2.52	abc	1.82	a
		Mc	1.91	a	1.70	bcde	2.53	abc	1.81	a
		Mo	1.91	a	1.70	cde	2.53	abc	1.76	a
		Mv	1.80	abc	1.64	e	2.51	abc	2.16	a
	Low Fertilizer									
		MM	1.93	a	1.71	bcde	2.52	abc	2.09	a
		MS	1.95	a	1.73	abcde	2.53	ab	1.84	a
		MC	1.94	a	1.67	cde	2.49	abc	1.88	a
		Mc	1.83	abc	1.65	e	2.51	abc	2.08	a
		Mo	1.91	a	1.68	cde	2.53	ab	2.09	a
		Mv	1.82	abc	1.69	cde	2.47	c	1.96	a
Reduced Tillage										
	High Fertilizer									
		MM	1.86	abc	1.65	de	2.52	abc	2.19	a
		MS	1.90	a	1.84	a	2.57	a	1.79	a
		MC	1.78	abc	1.76	abcd	2.57	a	2.18	a
		Mc	1.87	ab	1.84	a	2.56	a	2.16	a
		Mo	1.66	c	1.77	abcd	2.54	ab	1.86	a
		Mv	1.87	ab	1.68	cde	2.56	ab	1.86	a
	Low Fertilizer									
		MM	1.68	abc	1.74	abcde	2.56	a	2.07	a
		MS	1.67	abc	1.78	abc	2.56	abc	2.09	a
		MC	1.90	a	1.67	cde	2.54	ab	1.71	a
		Mc	1.89	a	1.66	de	2.54	ab	1.59	a
		Mo	1.77	abc	1.83	a	2.55	ab	1.90	a
		Mv	1.89	a	1.82	ab	2.55	ab	1.73	a
		LSD $_{(P = 0.05)}$	*0.20*		*0.12*		*0.06*		*0.63*	

[#]2011/2012 was inoculated with mycorrhiza, Abbreviation: MM = maize monoculture; MS = maize soybean rotation; MC = maize cowpea rotation; Mc = maize cowpea intercropping; Mo = maize oats intercropping; Mv = maize vetch intercropping. LSD = Least significant difference within season with P value = 0.05.

Table 3.5 Analysis of variance results for EEG concentration in mg/g soil for all growing seasons.

Source of Variation	df	Growing Seasons							
		2008/2009		2009/2010		2010/2011		2011/2012	
		Mean Square	P value	Mean Square	P value	Mean Square	P value	Mean Square	P value
Block stratum	2	0.86	NS	0.17	NS	0.05	NS	1.70	NS
Tillage	1	0.11	NS	0.07	NS	0.02	NS	0.02	NS
Error (a)	2	0.08		0.11		0.00		0.17	
Fertilizer application	1	0.00	NS	0.00	NS	0.00	NS	0.00	NS
Tillage X Fertilizer application	1	0.00	NS	0.00	NS	0.00	NS	0.19	NS
Cropping System	5	0.01	NS	0.01	NS	0.00	NS	0.07	NS
Tillage X Cropping systems	5	0.03	NS	0.01	NS	0.00	NS	0.08	NS
Fertilizer application X Cropping systems	5	0.01	NS	0.02	**	0.00	NS	0.12	NS
Tillage x Fertilizer application X Cropping systems	5	0.03	NS	0.01	NS	0.00	NS	0.14	NS
Error (b)	44	0.02		0.01		0.01		0.14	
Total	71								

[#]2010/2011 was inoculated with mycorrhiza. NS = not significant, ** = P ≤ 0.01.

3.5 AM fungal root colonization

Microscopic examination of stained maize roots from the first two growing seasons revealed only the presence of non-AM fungal structures. The structures observed were dark brown and reddish septate hyphae that were most probably those of a soil-borne root pathogen (Fig 3.4). The presence of these septate hyphal structures indicated poor infectivity of the crops by AM fungi. As a result, the decision to apply an inoculum (Mycoroot) in third growing season was made. Assessment of stained roots 8 weeks after field inoculation for growing season 2010/2011 revealed successful root colonization by AM fungi. Mycorrhizal colonisation was confirmed by the formation of intracellular hyphae, vesicles, and spores (Fig 3.5), although few arbuscules were detected in the selected root pieces examined. Root colonization assessment data for season 3 and 4 are shown Table 3.6 and 3.7. Notable by their absence, the soil borne fungal structures noted in the first two seasons (Fig 3.4) were not observed.

Percentage root colonization after inoculation reached a grand mean of 63%, this was not continued into the following season where percentage colonisation was only 2% (Table 3.5).

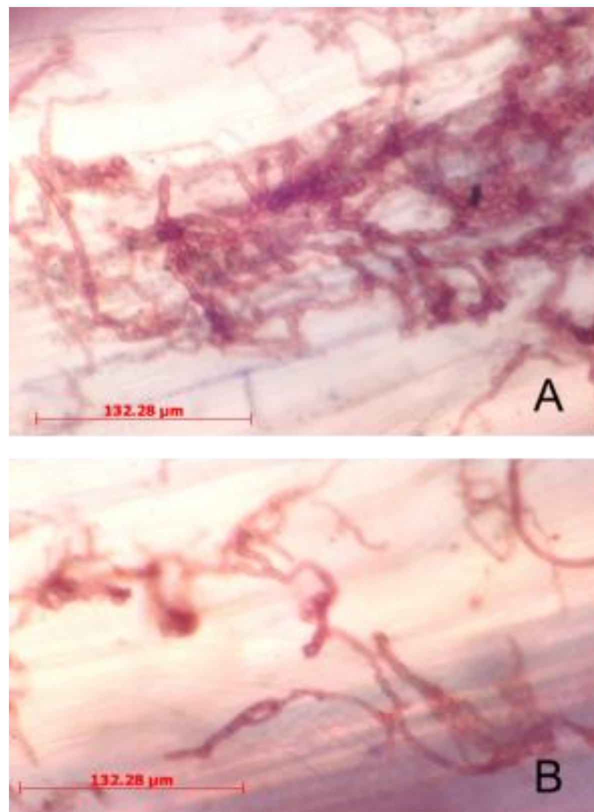

Figure 3.4 Maize roots from growing season 2008/2009 and 2009/2010 colonized with brown reddish hyphal networks (A and B).

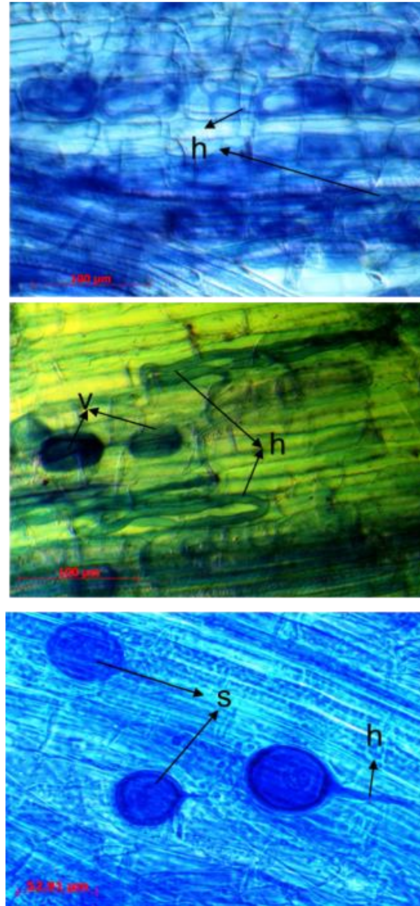

Figure 3.5 Maize roots after mycorrhizal inoculation (A) intra-radical hyphal networks (B) intra-radical vesicles (V) attached to hyphal networks (C) spores (S) with hyphal attachment (h).

Neither tillage, fertilizer nor cropping system had any significant effect on AM fungal root colonisation in season 3 or 4 (Table 3.6). The unprotected LSD analysis shows that colonisation percentage was reduced under conventional tillage and low input fertilizer application season (Table 3.6). On the other hand, root colonisation increased under reduced tillage, maize cowpea rotation and maize oats delayed intercropping (Table 3.6). However, colonisation decreased under conventional tillage, with both high and low input fertilizer applications.

The Mycoroot inoculum was not applied in season 4 and percentage colonisation dropped dramatically (Table 3.6) to levels close to 0% as observed in the first two season. Plots with high input fertilizer and cropping system with maize oats delayed intercropping and reduced tillage with high input fertilizer. Overall, maize oats delayed intercropping samples had the highest percentage colonisation of 5-7% (Table 3.6). There was no significant effect of all cropping systems on AM root colonisation for growth season three and four (Table 3.7).

Table 3.6 Mean values of AM fungal root colonisation

Tillage	Fertilizer	Cropping system	Season 1		Season 2		Season 3		Season 4	
			2008/2009		2009/2010		2010/2011#		2011/2012	
Grand Mean			0		0		63		2	
Tillage										
Conventional Tillage			0		0		67	a	1	a
Reduced Tillage			0		0		60	a	2	a
		P value					*0.39*		*0.25*	
Fertilizer										
	High Fertilizer		0		0		66	a	2	a
	Low Fertilizer		0		0		60	a	2	a
		P value					*0.08*		*0.84*	
Cropping system										
		MM	0		0		21	a	2	a
		MS	0		0		26	a	1	a
		MC	0		0		18	a	2	a
		Mc	0		0		21	a	1	a
		Mo	0		0		27	a	3	a
		Mv	0		0		20	a	1	a
	Unprotected LSD	*(P = 0.05)*					*11*		*3*	
Tillage X Fertilizer										
Conventional Tillage										
	High Fertilizer		0		0		57	ab	2	a
	Low Fertilizer		0		0		61	b	1	a
Reduced Tillage										
	High Fertilizer		0		0		63	ab	2	a
	Low Fertilizer		0		0		70	ab	2	a
	Unprotected LSD	*(P = 0.05)*					*9*		*2*	
Tillage X Cropping System										
Conventional Tillage										
		MM	0		0		65	ab	2	ab
		MS	0		0		53	b	0	b
		MC	0		0		55	ab	1	ab
		Mc	0		0		61	ab	1	ab
		Mo	0		0		61	ab	1	ab
		Mv	0		0		62	ab	0	b
Reduced Tillage										
		MM	0		0		66	ab	3	ab
		MS	0		0		64	ab	1	ab
		MC	0		0		63	ab	3	ab
		Mc	0		0		70	a	0	b
		Mo	0		0		71	a	5	a
		Mv	0		0		67	ab	2	ab
	Unprotected LSD	*(P = 0.05)*					*16*		*4*	

2011/2012 was inoculated with mycorrhiza, Abbreviation: R = reduced tillage and C = conventional tillage, FL = fertilizer low input; FH = fertilizer high input; MM = maize monoculture; MS = maize soybean rotation; MC = maize cowpea rotation; Mc = maize cowpea intercropping; Mo = maize oats intercropping; Mv = maize vetch intercropping. LSD = Unprotected least significant difference within season with P value = 0.05.

Table 3.6 Continued:

Tillage	Fertilizer	Cropping system	Season 1 2008/2009		Season 2 2009/2010		Season 3 2010/2011#		Season 4 2011/2012	
Fertilizer X Cropping System										
	High Fertilizer									
		MM	0		0		62	a	3	ab
		MS	0		0		56	a	0	b
		MC	0		0		56	a	0	b
		Mc	0		0		60	a	1	ab
		Mo	0		0		62	a	5	a
		Mv	0		0		66	a	0	b
	Low Fertilizer									
		MM	0		0		69	a	2	ab
		MS	0		0		61	a	1	b
		MC	0		0		62	a	4	ab
		Mc	0		0		71	a	0	b
		Mo	0		0		70	a	1	ab
		Mv	0		0		63	a	2	ab
Unprotected LSD *(P = 0.05)*							*16*		*4*	
Tilage X Fertilizer X Cropping system										
Conventional Tillage										
	High Fertilizer									
		MM	0		0		64	ab	2	b
		MS	0		0		55	ab	0	b
		MC	0		0		50	b	0	b
		MC2	0		0		54	ab	2	ab
		Mo	0		0		57	ab	2	ab
		Mv	0		0		64	ab	0	b
	Low Fertilizer									
		MM	0		0		67	ab	3	ab
		MS	0		0		51	b	0	b
		MC	0		0		61	ab	2	b
		MC2	0		0		67	ab	0	b
		Mo	0		0		65	ab	0	b
		Mv	0		0		60	ab	0	b
Reduced Tillage										
	High Fertilizer									
		MM	0		0		60	ab	4	ab
		MS	0		0		57	ab	1	b
		MC	0		0		64	ab	1	b
		MC2	0		0		65	ab	0	b
		Mo	0		0		67	ab	7	a
		Mv	0		0		68	ab	0	b
	Low Fertilizer									
		MM	0		0		71	ab	1	b
		MS	0		0		71	ab	2	b
		MC	0		0		62	ab	5	ab
		MC2	0		0		74	a	0	b
		Mo	0		0		74	a	2	ab
		Mv	0		0		65	ab	4	ab
Unprotected LSD *(P = 0.05)*							*23*		*5*	

2011/2012 was inoculated with mycorrhiza, Abbreviation: R = reduced tillage and C = conventional tillage, FL = fertilizer low input; FH = fertilizer high input; MM = maize monoculture; MS = maize soybean rotation; MC = maize cowpea rotation; Mc = maize cowpea intercropping; Mo = maize oats intercropping; Mv = maize vetch intercropping. LSD = Unprotected least significant difference within season with P value = 0.05.

Table 3.7 Analysis of variance of AM fungal percentage root colonization

Source of Variation	Growing Seasons 2010/2011 *df*	Mean Square	P value	2011/2012 *df*	Mean Square	P value
Block stratum	2	2334.71	NS	2	2334.71	NS
Tillage	1	793.80	NS	1	793.80	NS
Error (a)	2	675.34		2	675.34	
Fertilizer application	1	579.07	NS	1	579.07	NS
Tillage X Fertilizer application	1	22.82	NS	1	22.82	NS
Cropping System	5	148.49	NS	5	148.49	NS
Tillage X Cropping systems	5	43.26	NS	5	43.26	NS
Fertilizer application X Cropping systems	5	68.67	NS	5	68.67	NS
Tillage x Fertilizer application X Cropping systems	5	66.21	NS	5	66.21	NS
Error (b)	43	184.50		41	9.39	
Total	70	4916.8		68	4741.7	

2010/2011 was inoculated with mycorrhiza, NS-not significant, * = $P \leq 0.05$, ** = $P \leq 0.01$.

4. DISCUSSION

4.1 AM fungal spores

In the soil, arbuscular mycorrhizal fungi produce propagules such as spores, colonised root fragments, soilborne auxillary vesicles (some genera) and hyphae as a source of inoculum for the completion of their life cycle (Jaspers *et al.*, 1993). The presence of these propagules can be used as a measure of the activity and infectivity of these fungi in the soil (Bedini *et al.*, 2007, Castillo *et al.*, 2006, Verzeaux *et al.*, 2017). Any changes in the propagule numbers can be a useful marker to indicate the effect of agricultural practices on soil health, with spores' enumerations being the most widely used measurement (Avio *et al.*, 2013, Aggarwal *et al.*, 2010, Boddington and Dodd, 2000, Duponnois *et al.*, 2001, Jansa *et al.*, 2003, Matimaran *et al.*, 2007).

Spore numbers enumerated in this study were low. It has long been recognised that the presence of low AM fungal spore numbers is directly related to soil disturbance (Jasper *et al.*, 1989). These findings are similar to several other studies (Avio *et al.*, 2013, Bedini *et al.*, 2007, Castillo *et al.*, 2007). The low number of spores recorded in this study are similar to another pilot study conducted in the Bophirima District in the Ganyesa sub-region of North West province of South Africa were low numbers of spores were also found as compared to natural, grassland and pasture soils (Ingleby and Dirk, 2006). On the contrary Castillo *et al.*, (2006) recorded counts ranging from 182 to 238 spores / 100 g of dry soil under conventional tillage, and 280 to 641 spores / 100 g under no-till. These findings confirm that different tillage practices have varying effects on the number of AM fungal spores because they create varying forms of disturbance in the soil chemical, physical and biological properties (Aggarwal *et al.*, 2010, Kabir *et al.*, 1998, Schalamuk and Cabello, 2010, Verzeaux et al., 2017).

Conventional tillage positively influenced AM fungal activity and this is evident as a high number of AM fungal spores was recorded under this treatment overall growing seasons. For that reason, we predict that several factors could have attributed to the findings. One of the contributing factors could be that these fungal communities could be well adapted to mould plough tilling management of the soil as it was previously practiced. Prior to the establishment of this study, the soil was intensively mould ploughed tilled and left fallow for five years. Therefore, this previous management practices could have attributed to the low number of spores. Generally, conventional tillage can reduce AM fungal spores because it dilutes and redistribute spores within the soil at different depths by the mixing of topsoil with below ground soil (Douds *et al.*, 1995, Franke-Synder *et al.*, 2001). The re-distribution of propagules through mixing of the soil reduces the chances of symbiotic association of spores with host crops (Wu

et al., 2016). Furthermore, conventional tillage can select certain types of AM fungal spores because spore differs in their resilience to disturbance by tillage. Some fungal spore can be affected by the physical disruption of their propagules which can make them dormant or useless. Other fungal spores can be negatively affected by the alteration of their life cycle by creation of unfavorable conditions that can affect their sporulation hence life cycle (Aggarwal *et al*., 2010, Douds *et al*., 1995, Kabir *et al*., 1998, Schalamuk and Cabello, 2010).

No significant differences in spore numbers between tillage practices after four growth seasons (Table 3.1) was recorded, given the low spore numbers, this was not unexpected. The slight non-significant increase in spore numbers under conventional tillage may be due to intensive soil preparation at the beginning of the trial that could have shifted propagule distribution by mixing of soil strata diluting spore concentrations (Douds *et al*., 1995; Franke-Synder *et al*., 2001, Galvez *et al*., 2001, Habte, 2000, Honermeier, 2007, Johnson and Pfleger, 1992) resulting in reduced effectiveness (Bethlenfalvay, 1992; Hamel, 1996). Soil preparation also results in destruction of hyphal networks that can alter the life cycle (Bethlenfalvay, 1992) and functionality of these fungi (Castillo *et al*., 2006, Douds *et al*., 1995). Increased spore numbers in the growth season 2010/2011 and 2011/2012 (Table 3.1) can be solely attributed to the application of inoculum which was applied to all plots. The results indicate that different management practices affect AM fungal spore number in different ways (Curaqueo *et al*., 2011, Noelia Cofré *et al*., 2017).

These results showed that tillage, high fertilizer application, and cropping systems did influence AM fungal spore numbers to some extent particularly in the 1st, 2nd and 4th growth seasons (Table 3.1). Results from the first two growth season may indicate that some adaptation to intensive management practices has occurred. It is well documented that certain practices such as tillage can result in the selection of certain AM fungal species (Jasper et al., 1993; Jansa *et al*., 2003). In a study by Galvez *et al*., (2001) they reported that *Glomus etunicatum* spores were abundant under chisel disk tillage while *G. occultum* spores were more abundant under no-till, and *G. geosproum* spores were not affected. Soil depth can also affect the distribution of AM spores. Abbot and Robson (1991) recorded higher spore numbers at 8 cm depth under tilled soil as compared to 8-15 cm depth. Soil samples in this study were collected from the top 20 cm and effect of depth was not taken into consideration.

Historical soil management practices may also be responsible for low spore numbers. The trial site was fallow for five years prior to establishment. Initially, this effect was not taken into consideration, but lack of suitable host plant material would have resulted in reduced spore numbers. Kabir *et al*., (1999) found decreasing number of AM fungal spores with increasing

length of fallow period from 86, 74 and 54 spores / 100 g soil after 30, 60 and 90 days fallow, respectively. Duponnois *et al.*, (2001) recorded spore numbers ranging from 116 to 419 spores / 100 g soil which decreased from 4 years fallow to 11 years fallow. Spores are known to be the longest surviving AM fungal propagules in the soil. Spores will germinate in the presence of various stimuli such as moisture and presence of a host plant. If host plant roots are not available germination will cease. Several germination attempts can be made but extended fallow period would result in depletion of spore carbon reserves resulting in the loss of viability (Brundrett, 2009, Tommerup, 1983 & 1984). Enumeration of spores after wet sieving does not account for viability, although every attempt was made to count only translucent, lipid-filled spores which may be viable. Under these conditions, inoculation can improve AM fungal populations and activity in soil and is recommended as good practice for degraded soils. In order to sustain mycorrhizal populations, successive inoculations may be required for a further 2 to 5 growth seasons under reduced tillage as shown by Pellegrino *et al.*, 2011 and Salami and Osonubi (2002).

This study indicated that crop rotation and intercropping with legumes positively affected the number of AM spore in the soil. AM fungi are intimately linked to their host plant and mycorrhizal status of host plants can affect spore numbers (Aggarwal *et al.*, 2010, Astiko *et al.*, 2016, Lekberg and Waller, 2016). This emphasises the need to consider mycorrhizal status of crops selected for crop rotation and intercropping in order to support and increase AM fungal diversity (Kahiluoto *et al.*, 2009, Mozafar *et al.*, 2000). It is important to note that sporulation is a survival mechanism for AM fungi, allowing it to overcome unfavorable conditions (Smith and Read, 2008). Agricultural management practices combined with other factors such as host plant and soil environmental conditions are determinants of the occurrence and spore numbers in this site and could account for the variability observed in this study. Kramadibrata *et al.*, (1995) in Indonesia recorded 383 to 12 266 spores / 100 g soil in soybean rhizosphere while Medina *et al.*, (1988) in Florida recorder 5 to 679 spores / 100 g soil under 4 forage legumes. The cropping systems most favorable in this study were maize vetch intercropping (1st and 4th growth season) and maize cowpea rotation (3rd growth season).

4.2 Identification of AM fungal taxa

In this study, three resident AM fungal spore morphotypes were tentatively placed in the genera *Scutellospora*, *Gigaspora,* and *Glomus*. This lack of diversity is directly related to the low recovery of spores from the soil, therefore, interactions with tillage, fertilization and cropping systems could not be ascertained. Similar results were reported by Bedini et al. (2007) who sampled soil cultivated to maize for more than 50 years in Italy. *Glomus* species are known to dominant agricultural soils (Bedini *et al.*, 2007, Oehl *et al.*, 2003, 2010,

Schalamuk and Cabello, 2010, Schalamuk *et al*., 2006) and *Gigaspora* species are not easily affected by soil disturbance (Hamel, 1996, Johnson and Pfleger, 1992). It is important to note that the presence of spores combined with the absence of colonisation in roots during the first two growth season (Table 3.6) indicated that spores were not viable.

A survey of indigenous populations of AM fungi in South Africa under intensively managed vineyards showed a prevalence of 5 genera (Meyer *et al*., 2005). In contrast Dames (1991) identified 13 species from natural savanna soils in the Limpopo province, South Africa and showed that the presence of individual species differed in their correlation to various soil factors such as nutrients and moisture. A study by Uhlmann *et al*., (2004) in the semi-arid region of Namibia showed that AM fungal communities were more affected by vegetation cover the rainfall regime than land use management systems. These studies were based on identifications made microscopically using limited spore morphological characteristics (Morton, 1988), the use of next-generation sequencing has been more recently used for population studies in South Africa. Moore (2015) using 454-Pyrosequencing revealed that the AM fungal community composition was dominated by species in the *Ambispora*, *Glomus* and *Paraglomus* genera with rarer components being represented by the genera *Redeckera*, *Archaeospora* and *Geosiphon* in soils from natural vegetation collected from Coega, Eastern Cape and Upington, Northern Cape. Eighteen operations taxonomic units were identified from Spekboom (*Portulacaria afra*) thicket in Eastern Cape, South Africa being composed of 7 species of *Paraglomus,* 3 of *Ambispora*, 1 each of *Claroideoglomus, Archaeospora* and *Geosiphon* and 5 *Glomus* (Dames and Fulmaka, 2015). Given the importance of the mycorrhizal relationship populations under different land use types in South Africa requires further attention to establish baseline populations.

4.3 Glomalin (EEG) quantification

Since glomalin is released into the soil when dead AM fungal hyphae and spores are deposited and decomposed, the concentration of this protein can be directly related to the activity of these fungi in the soil (Driver *et al*., 2005; Rillig *et al*., 2003, Steinberg and Rillig, 2003, Wright and Upadhyaya, 1998 and 1999, Wright *et al*., 2006). The term glomalin is currently used to refer to the purified protein while 'glomalin-related soil protein' (GRSP) is commonly used to refer to the soil extract (Bedini *et al*., 2007, Fokom *et al*., 2012, Preger *et al*., 2007, Rillig, 2004, Rillig *et al*., 2003; Wright *et al*., 2006 and 2007). The fraction extracted in this study is referred to as easily extracted glomalin (EEG). The presence of this protein has been used as a bio-indicator of mycorrhizal activity under different agricultural management practices (Garciá-González et al., 2016, Preger *et al*., 2007, Rillig *et al*., 2003; Wright *et al*., 1998, 1999).

The concentration of EEG over the four growth seasons ranged on average from 1.72 and to 2.53 mg / g (Table 3.4). The low levels of EEG in the first two growth season could be the result of low AM spore numbers and absence of colonisation recorded in this study. The production, decomposition and standing stock of EEG in soil is directly linked to the presence of living and dead AM fungal hyphae and spores (Driver *et al.*, 2005, Rillig *et al.*, 2003, Wright et al., 2006).

The highest EEG concentrations were recorded in the third growth season (2010/2011) after inoculation and was significantly increased in reduced tillage plots (Table 3.4). The values recorded are similar to other studies. In apple orchards, Western Cape, South Africa Meyer *et al.*, (2015) reported overall mean EEG values of 1.73 and 1.52 mg/g in spring and summer, respectively. In a tropical rain forest landscape, Lovelock *et al.* (2004) reported EEG concentrations ranging between 1 and 8 mg/g of soil. Wright *et al.* (2007) recorded EEG concentrations within the ranges of 2.5-15 mg/g in undisturbed Ultisols soils in the Mid-Atlantic state area, with mean values of 2.86, 2.27, 2.09 mg/g of soil under no-till, conventional tillage and organic farming, respectively. Knorr *et al.* (2003) found average concentrations of 0.67 and 1.28 mg/g soil in 1994, and 0.81 and 1.28 mg/ g soil in 2000, with no significant changes in the GRSP after 6 years of sampling. Time is a critical factor when increased concentrations are the goal as it may require several years. The production of glomalin is dependent on an active population of AM fungi which deposits the protein in the soil on degradation (Rillig 2004).

There was a significant difference in EEG concentration for the 2nd and 3rd growth seasons with reduced tillage contributing significantly to an increase in EEG when compared to conventional tillage (Table 3.4). The relationship between glomalin and tillage has been reported in other studies (Borie *et al.*, 2006, Bedini *et al.*, 2007, Lee and Eom, 2009, Preger *et al.*, 2007, Rillig *et al.*, 2003, Wright and Anderson, 2000, Wright *et al.*, 1999 and 2007). Low levels of EEG, which represents newly deposited glomalin, is a good indication that no production has taken place for a long time as a result of lack of mycorrhizal activity (Lovelock et al., 2004). There was a significant difference in EEG concentration for 2nd growth season (2009/2010) under the interaction of fertilizer application and cropping systems (Table 3.5). Under high fertilizer application Maize Soybean rotation and Maize cowpea intercropping treatments increased EEG, while under low fertilization Maize Cowpea rotation and Maize cowpea intercropping has lowest EEG concentrations. It is speculated that microbial decomposition under low fertilizer was reduced. Little is known about the decomposition rate of glomalin as the protein is recalcitrant and can accumulate in the soil over a long period of time (Rillig, 2004). Meyer *et al.*, (2015) found no significant differences in EEG concentration in apple orchard soils, even when rye and oats were planted as cover crops in the work rows.

Unlike the current study, the presence of mycorrhizal activity as indicated by percentage root colonisation was evident in apple orchards, which resulted in continuous production of glomalin.

4.4 AM fungal root colonization

Lack of AM fungal structures such as hyphae, vesicles, and arbuscules from stained roots for the first two growth seasons indicated that AM fungal infectivity of field crops was non-existence. The presence of a soil borne fungus inside roots was noted but not identified. As mentioned previously the extensive fallow period before trial establishment could have resulted in reduced number of AM propagules in the soil. This was supported by the MPN results which indicated low propagule number in soil before inoculation, the only exception being the Mv intercropping treatment which supported higher propagule numbers. Despite this colonisation was not observed in these treatment plots. Fallow combined with the application of Roundup (glyphosate) may have limited AM colonisation. Recent research has shown that the application of glyphosate does reduce AM fungal spore viability and percentage root colonisation thus negatively affecting mycorrhizal functionality (Druille *et al.*, 2013a and 2013b). A maize pot trial conducted in collaboration with University of Fort Hare, South Africa indicated that after one application of glyphosate percentage AM colonisation was reduced by 30-50% depending on application rate (Dames personal communication). In this current trial, the plot received a minimum of three applications of glyphosate and another herbicide Dual S Gold (active ingredient S-metolachlor), the effect of this second herbicide on AM fungi is unknown. No interactions were recorded between the various agricultural management practices with AM fungal root colonization percentage in this study after the application of inoculum which was applied to the entire trial site to stimulate mycorrhizal activity in the third growth season (Table 3.7). Reduced tillage and low fertilization did significantly increase AM colonisation when compared to conventional tillage and high fertilization. Reduced tillage combined with Maize cowpea or Maize oats intercropping increased colonisation when compared to Maize Soybean rotation under conventional tillage (Table 3.6). Root colonization has been shown to be affected by agricultural practices, Jansa *et al.*, (2003) found higher root colonization under no-till than ploughed and chiselled soil. It is suggested that reduced infectivity of propagules could be the result of the reduction in propagules number through intensive dilution by tilling, or by changes in the soil environment (Habte, 2006, Njira et al., 2017), but many interacting factors could be at play. Lack of colonization in the first two growth seasons may have resulted from other factors not considered in this study such as soil type, environmental conditions and host plant type (Oehl *et al.*, 2010, Casazza *et al.*, 2017). From previous studies, it is reported that root colonization can be influenced by AM fungal species, soil type, agricultural practices, crop types and environmental factors such as pH, temperature

and soil moisture (Aggarwal *et al.*, 2010, Oehl *et al.*, 2010). As a result, certain species are adapted to performing well under certain soil conditions and if any changes occur to this soil conditions, this fungal species will no longer be active (Aggarwal *et al.*, 2010).

Increased percentage AM root colonization and overall increased propagule numbers after inoculum application indicates that inoculation encourages activity and infectivity of these fungi especially in degraded soil. The absence of the suspected soil borne root pathogen confirmed that the colonization of host roots by AM fungi effectively discourages pathogens. Several studies have shown that mycorrhizal root colonization of host plants controls the development and severity of root borne diseases (Gosling *et al.*, 2006, Mummey *et al.*, 2009, Upadhyaya *et al.*, 2010, Casazza *et al.*, 2017) especially under no-till or reduced tillage (Vos *et al.*, 2012) and enhances host fitness due to early symbiotic benefits to the host (Karasawa *et al.*, 2002, Martinez and Johnson, 2010, Pellegrino *et al.*, 2011). This emphasises the importance of AM fungi in protecting their host against pathogens attack (Garcia-Garrido *et al.*, 2000, Ozgonen *et al.*, 2010, Vos *et al.*, 2012, Mustafa *et al.*, 2016).

Similarly, root colonisation levels of between 70 % and 90 % were found in vineyards that were previously inoculated with commercial AM fungal inoculum at two farms in the Stellenbosch region of South Africa (Meyer *et al.*, 2005). Meyer *et al.*, (2015) also reported mean colonisation of un-inoculated apple roots of 50.8 and 67/1% in spring and summer, respectively. Jansa *et al.*, (2008) found 50 % of root length of medic and leek colonised within 4 weeks by *G. mosseae* and *G. intraradices*, he also found that *G. mosseae* colonised root faster than *G. intraradices* although root colonization levels were similar at 8 weeks. It is also important to note that different AM fungal species will exhibit differences in infectivity with some species such as those belonging to the Glomeraceae colonizing roots within 4 weeks while members of the Acaulosporaceae and Gigasporaceae are slow colonizers requiring about 8 weeks (Hart and Reader, 2002b). The inoculum applied in the current study container several indigenous strains of AM fungi (Appendix C). Root colonisation was subsequently drastically reduced in the 4th season (Table 3.6), the inoculum was not reapplied. AM spores were present in the soil after the previous season inoculation and one can only speculate that reduced colonisation was a result of the effect of herbicide treatments, particularly glyphosate as already discussed.

The most propagules number method was used to determine the infectivity of AM fungi as a measure of their activity in this study. Although this method can underestimate the presence of infective propagules it does emphasise the importance of viable propagules (Mathimaran *et al.*, 2007, Njira *et al.*, 2017). This method does not consider one inoculum source above

another. Thus, the use of selective inoculum can select certain AM fungal species because some species depend on some propagules more than others. Although mostly low (Table 3.3) colonisation was still recorded indicating that adverse field effects may have been negated as a result of the soil dilution. AM fungal activity is also dependent on triggers that assist in the recognition of host crops, the use of sorghum as a standard host plant could indicate a better host recognition response under the controlled conditions of the test (Bansal *et al.*, 2012, Borriello *et al.*, 2012). These findings support the theory that there is preferential establishment of the symbiotic association between certain plants and AM fungal species (Mathimaran *et al.*, 2007, Janouškova *et al.*, 2017).

These findings further supported the AM fungal propagules adaptation to previous management practices such as bare fallow periods. This is shown by the establishment pot cultures several months after incubation of the soil. The soil samples were under stored room temperature for six months before the establishment of pot cultures under controlled environmental conditions. The storage of this soil was almost similar to bare fallow periods previously used to manage the soil in the study site. As a result, the storage or incubation could have created favorable conditions that promoted sporulation and hyphal growth that contributed to the mycorrhizal infectivity and activity. This is similar to other studies that have used incubation of soil inoculum to activate or maintain activity of AM fungi. The theory is that some spores require longer periods of incubation for activation of sporulation and infectivity after germination of their spores (McGee, 1989, Tommerup, 1983 and 1984). However, other factors like growth season, crop growth stages, and environmental conditions (pH, nutrients, and temperature) do also play a role in the infectivity of host crops by AM fungi under field conditions and pot cultures. The development of tolerance to management practices can also influence spore not to become dormant. Consequently, change in the environmental conditions could have contributed to the establishment of infectivity under trap culture. We are expecting these to increase due to increased inoculum that will be produced by inoculated species. Increased infectivity resulted after inoculation proving that AM fungi inoculation is important, to increase mycorrhizal activity that will improve and maintain soil health status.

5. CONCLUSION

This study was part of a broader multidisciplinary conservation agriculture investigation conducted by the Agricultural Research Council (Report GW/A/2009/24, 201/24, 2011/24) parameters measured included soil and leaf nutrient analysis and soil enzyme activity. Factors generally positively correlated (coefficient of 0.6 and above) to AM fungal parameters (EEG, Spore numbers, and percentage root colonisation) over the first 3 growth seasons were

Topsoil NH_4, Topsoil C, soil enzyme activity (alkaline and acid phosphatase, urease and β-glucosidase).

Over the four growth seasons (2008/2009, 2009/2010, 2010/2011 and 2011/2012) since the establishment of this trail regardless of the low number of spores, EEG concentrations and no evidence of root colonization from background population, statistical differences between reduced and conventional tillage and the sub-treatments (FL = fertilizer low input; FH = fertilizer high input; MM = maize monoculture; MS = maize-soybean rotation; MC = maize cowpea rotation; Mc = maize cowpea intercropping; Mo = maize oats intercropping; Mv = maize vetch intercropping) at a 95% confidence level were detected, but it is too early to make conclusive deductions. The results obtained from this study provide a baseline data set for this key fungal group and will serve as future reference. Inoculation of this field successfully re-introduced AM fungi after 8 weeks and increased spore numbers and root colonization was recorded. This emphasises the importance of inoculation with AM fungi for improving soil health, particularly when background populations have been significantly reduced. Therefore, in order to maintain mycorrhizal populations after inoculation one could combine reduce tillage and intercropping of mycorrhizal dependent perennial host plants like annual rye grass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), cowpea, soybean or *Sorghum* species. This will provide sufficient root material for the mycorrhizal fungi to continue to propagate in the soil.

Crop rotation and intercropping with highly mycorrhizal crops are good practices for maintaining high AM fungal activity in the soil (Ibijbijen *et al.*, 1996, Kahiluoto *et al.*, 2009, Mozafar *et al.*, 2000, Njira *et al.*, 2017), but this was not evident in this study emphasising the need to assess the mycorrhizal potential of the soil to determine whether inoculation is required. This can be achieved but conducting infectivity tests using MPN methodology or through spore counts, bearing in mind that not all spores may be viable. Inoculating with AM fungi increases spore number and colonization of crops in the field, resulting in increased mycorrhizal activity that in the long term will contribute in building up soil aggregates and stabilizing soil structure through hyphal networks (Hamel, 1996, Jeffries *et al.*, 2003, Wright *et al.*, 1999) and production of a glue-like glycoprotein, glomalin in the soil (Bedini *et al.*, 2007, Gosling *et al.*, 2006, Harrier, 2001, Lutgen *et al.*, 2003, Schloter *et al.*, 2003, Wright and Andersen, 2000). The longer-term effect of inoculation on establishing populations was not the scope of this study but subsequent inoculations would greatly improve re-establishment of fungal populations.

The enumeration of AM fungal spores from soil samples show that background populations was low, with little or no activity in the soil. Estimation of AM soil infectivity indicated that the background population in the soil was unable to form symbiotic association with the crops that were planted. Quantification of EEG indicated that there was little production of glomalin in this soil. Estimation of spores and their infectivity, determination of species type and composition, and quantification of EEG correlate, and all these parameters indicated that AM fungi were not actively operating in this soil.

The low number of spores, with no root infectivity of background population, resulted in the inability to establish pot cultures required for taxonomy and identification of species. To improve on these results determination of AM fungal species with molecular identification of both field and pot cultures with new and more sensitive techniques such as PCR based methods with taxon and species-specific primers (Johansson *et al*., 2004, Ma *et al*., 2005, Liang et al., 2008). Studies should further include estimation and enumeration of other propagules such as hyphae. Determination of root infection must include measurements of other contributing factors like environmental factors such as temperature, soil moisture, and pH. Quantification of AM fungi must include determination of glomalin with more sensitive immunological methods using the specific monoclonal antibody, MAb32B11. Changes to the populations and further diversity studies should also involve more sensitive techniques such as 454-pyrosequencing and identification of operational taxonomic units (Kruger *et al*., 2012).

AM fungi are indicators of soil health under different agricultural practices and confirm that these fungi are very sensitive to any changes to soil properties (Schalamuk and Cabello, 2010). It is therefore important to manage soil with these fungi in mind in order to support active populations that will positively influence the soil physical, chemical and biological properties including plant health.

In conclusion, agricultural studies in disturbed soil needs to be restored, maintained, sustained and monitored by high mycorrhizal activity that is interlinked with soil chemical, physical and biological components. It is recommended that future agricultural management of soil must include the monitoring of AM fungi spore number, activity, infectivity, species composition, community structure, diversity and glomalin production, deposition and standing stocks in the soil as they positively influence soil physical, chemical, and biological properties. Similar studies to the one conducted should be used to further investigate the role of AM fungi as bio-indicators under agricultural soil in South Africa. Therefore, sustainable agriculture management practices cannot be practiced without AM fungi, because they are good bio-indicator of soil health under different agricultural practices in South Africa. Therefore, AM

fungal activity, spore numbers, abundance, and production of glomalin are essential in indicating changes in soil chemical, physical and biological properties of soil in agricultural management fields (Anguacil *et al*., 2008, Castillo *et al*., 2006) and they are well established measures of soil health under changing conditions.

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APPENDIX A: REAGENTS

AM Fungal Spore Extraction (Smith and Dickson, 1997)

Sucrose solution for spore extraction (60%)

600 g sucrose

1000 ml of distilled water (dH_2O)

Ethanol for storing roots (50 %)

1000 ml Ethanol

1000 ml of dH_2O

KOH for clearing roots (5%)

100g of KOH

2L dH_2O

Alkaline peroxide for bleaching roots

3 ml NH_4OH

30 ml 10% (v/v) H_2O_2.

567 ml dH_2O

0.1 M HCl for acidifying the roots

22.79 ml HCl

2 L dH_2O

Lactoglycerol Trypan blue stain for staining roots

520 ml lactic acid

480 ml glycerol

640 ml dH_2O

0.82 g trypan blue

Lactoglycerol for destaining roots

520 ml of lactic acid

480 ml glycerol

640 ml dH_2O

APPENDIX B: STAINS

(Morton, 1988)

Polyvinyl PVGL for mounting roots on slides

100 ml of lactic acid

100 ml of glycerol

16.6 g of polyvinyl alcohol

100 ml of dH_2O

Melzers stain for staining and mounting roots

15 g Potassium iodide

0.5 g iodine stain

100g chloral hydrate

22 ml dH2O

APPENDIX C: MYCOROOT AM FUNGAL SPECIES COMPOSITION

The Mycoroot SuperGro inoculum product contained the listed species that were identified with morphological and molecular methods

AM fungal Species	Number of spores/g of inoculum	Infectivity of propagules
Glomus clarum *Glomus mosseae* *Glomus etunicatum* *Gigaspora gigantea* *Paraglomus occultum*	10 spores/g of soil	80-100 propagules/g of inoculum

APPENDIX D: [BSA standards] graph